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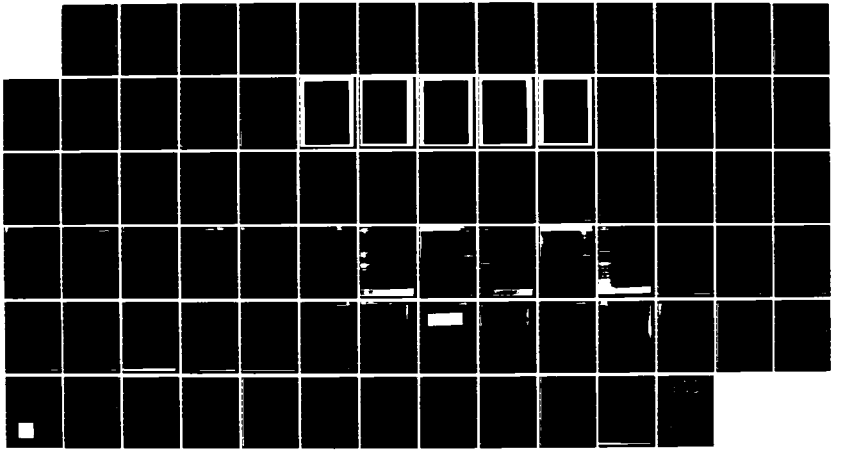

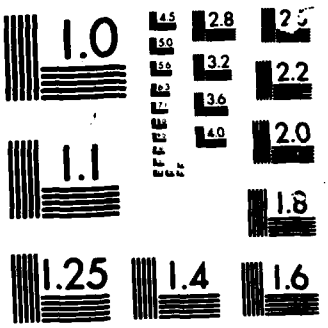

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Safety and Immunogenicity Testing of a Pilot Polysaccharide Vaccine Preparation to Pseudomonas aeruginosa

FINAL REPORT

Gerald B. Pier, Ph.D.

February 15, 1985

SUPPORTED BY

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We prepared and tested a high-molecular weight polysaccharide antigen from three different immunotype strains of Pseudomonas aeruginosa. These materials were tested in humans for safety and immunogenicity. All three vaccines were found to be safe in doses between 100-250 mcg. They were also found to be immunogenic, inducing antibody responses in 40-90% of immunized persons. These antibodies were also found to be functional in their ability to kill the bacterim in conjunction with white blood cells. In addition, we showed that these vaccines were able to induce an effect on T-cells of immunized animals and humans. This T-cell response may be an important componen of the overall immune response to these antigens that results in protective immunity.

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FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

A. Contract Background

The principal investigator has been conducting research under a contract from the U.S. Army Medical Research and Development Command (USAMRDC) that ran from 1 March 1979 to 31 March 1984. The work to be conducted under this contract included preparation and testing of polysaccharide vaccines to Pseudomonas aeruginosa. This research included phase 1 human trials of the vaccines, development of assays to measure antibody responses of immunized humans and infected animals, and assessment of the vaccine potential of the material in animal models of P. aeruginosa infection. Other studies included determinations of cellular responses to vaccination, determination of the genetic basis of the response to the vaccine in inbred mouse strains, and assessment of the role of lipopolysaccharide (LPS) as an immunogenic or biologically active component of the vaccine. The vaccine under contract is a high molecular weight polysaccharide (PS) material isolated from the outer cell surface or culture supernate of P. aeruginosa. Similar types of polysaccharides have been shown to be effective vaccines for a number of bacterial infections, such as meningitis caused by Neisseria meningitidis and pneumonia caused by Streptococcus pneumoniae. Since P. aeruginosa infections are common complications of wound and burn injuries that occur in military combat, the PS vaccine under development could have potential as a preventative measure for these infections.

B. Results on Vaccine Testing

Under this contract we prepared and tested high molecular weight polysaccharide antigens in phase 1 safety and immunogenicity trials of healthy humans. The polysaccharide antigens were prepared from three different immunotypes strains of P. aeruginosa, immunotypes 1, 2, and 3. Results regarding the human immune response to the immunotype 1 high molecular weight PS have been published. We found that doses of 100-250 ug of PS induced a brisk antibody response. The antibody made was able to kill P. aeruginosa in an opsonophagocytic assay. The antibody was found to be principly of the IgG class, although IgA and IgM responses were also made. Similar findings have occurred with the PS vaccine prepared from the IT-2 strain. Approximately 15 people have been immunized with this material. All persons increased their antibody levels by four-fold or greater two weeks following immunization. All persons immunized also increased their opsonophagocytic killing titer by four-fold or more. Both the IT-1 and IT-2 vaccines were very safe in humans, eliciting no greater than a slightly sore and tender arm at the sight of injection. The IT-3 vaccine has been given to 8 persons. Three of the eight persons have made an immune response to this material while the other 5 did not. All persons were given a 100 ug dose. The reasons for this poor response are unclear and are currently under investigation. However, the problem does not appear to be in the vaccine but rather in the genetic capabilities of humans to respond to this material.

We have also analysed the mitogenic response of immunized individuals to these PS materials. We have found that persons immunized with the IT-1 and IT-2 PS vaccine have cellular proliferative responses when peripheral blood lymphocytes are incubated in culture with purified PS. Among immunized persons this appears to be a T cell immune response.

Among non-immune persons there is either a low level mitogenic response, or B cell response. These data suggest that immunization of humans with a high molecular weight PS induce a T cell response.

All of the remaining aspects contracted for by the USAMRDC have been published. The findings have been previously submitted to the USAMRDC, and are included in the final report as part of the bibliography.

C. Personnel receiving contract support:

1. Gerald B. Pier, Ph.D.; Robert Gillette, B.A.; Mark Cohen, B.A.; Mary Ellen Elcock, B.A.

D. Chronological Bibliography

1. Pier GB, Markhan RB, Eardley DD. Correlation of the biological response of C3H/HeJ mice to endotoxin with the chemical and structural properties of the lipopolysaccharides from Pseudomonas aeruginosa and Escherichia coli. J Immunol. 1981; 127:184-191.

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Final Inventory of Government Property Remaining

Recommendation for disposition:

The equipment bought under Army Contract DAMD17-79-C-9050 currently being be used by Dr. Dennis L. Kasper under under contract No. DAMD17-83-C-3239, "Development of vaccines to prevent wound infections." Dr. Kasper's laboratory is situated next to Dr. Pier's. All of the equipment purchased under contract No. DAMD17-79-C-9050 was needed by Dr. Kasper for his Army contract work. Therefore, we would like to recommend that the equipment bought under this contract remain in the possession of the Brigham and Women's Hospital in order that Dr. Kasper may continue to use this equipment to carry out the goals of his Army contract work.

Equipment Purchased on Army Grant 2-9-9984

Vendor	Quantity	Equipment
LKB	1	2070-001 Ultrorac 2 Fraction Collector
	1	2138-001 Uvicord S Flow Monitor
	1	6520-7 Six Channel DC Recorder
	1	2117-S3 System Three Multiphor
	1	2103-010 DC Power Supply
	1	2209-001 Cooling System
	1	2117-111 Surface Electrode for Radiometer pH meter 326
Waters Assoc.	1	48502-R404 Refractive Index Monitor
The London Co/ Rainin	1	TTT-60 Titrator Module
	1	PHM62SA pH Meter with Standard Access

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The London Co/ Rainin	1	MNV 1 Magnetic Valve
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CORRELATION OF THE BIOLOGIC RESPONSES OF C3H/HEJ MICE TO ENDOTOXIN WITH THE CHEMICAL AND STRUCTURAL PROPERTIES OF THE LIPOPOLYSACCHARIDES FROM *PSEUDOMONAS AERUGINOSA* AND *ESCHERICHIA COLI*[1]

GERALD B. PIER,[2] RICHARD B. MARKHAM,[3] AND DIANE EARDLEY

From The Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, and Harvard Medical School, and The Department of Microbiology, Harvard School of Public Health, Boston, MA

The basis of the biologic responses of C3H/HeJ mice to endotoxin administration in relation to the structural linkages in the lipid A portion of the lipopolysaccharide (LPS) of *Pseudomonas aeruginosa* and *Escherichia coli* were investigated. *P. aeruginosa* LPS was found to be immunogenic, mitogenic, and toxic, but not lethal, in C3H/HeJ mice. The observed mitogenicity in spleen cells was directed toward immunoglobulin- (Ig) bearing cells, was present in response to isolated and solubilized lipid A, and was inhibitable by polymixin B. The *P. aeruginosa* LPS was chemically analyzed in order to define its composition and exclude the presence of contaminating proteins being responsible for the biologic responses of C3H/HeJ mice that were observed. Structural analysis of the linkages of the fatty acids to the glucosamine backbone in the lipid A of *P. aeruginosa* and *E. coli* revealed similarities in terms of the ratio of hydroxy fatty acids to straight chain fatty acids and the way in which these 2 types of fatty acids were linked to the backbone. Differences were seen in the carbon chain length of the fatty acid substituents, and the substituent on the hydroxy fatty acid that is directly ester linked to the glucosamine backbone. These data indicate that the refractivity of C3H/HeJ mice to the biologic effects after the administration of Gram-negative endotoxins may be limited to enterobacterial LPS. Those differences we found in the chain length and/or linkages of the fatty acid substituents in the lipid A portion of the LPS between *P. aeruginosa* and *E. coli* may be sufficient to render C3H/HeJ mice responsive to the biologic effects of nonenterobacterial endotoxins.

The refractivity of C3H/HeJ mice to the biologic effects of lipopolysaccharide (LPS) or endotoxin from enterobacteriaceae is well documented (1–5). Among these defective responses are the low and transient nature of the primary antibody response to the LPS (2, 6), lack of induction of mitogenesis in spleen cell cultures (7), and refractivity of these mice to the toxic and lethal properties of LPS (1). Recently Spellman and Reed (8) and Moreno and Berman (9) have shown that C3H/HeJ mice are responsive to the biologic effects of *Brucella abortus* LPS. Although there are some similarities of the LPS from all Gram-negative organisms, the chemical composition and the biologic properties of the *B. abortus* LPS differs markedly from that of the enterobacterial LPS (10, 11). These chemical and biologic differences may readily explain C3H/HeJ mouse responsiveness to *B. abortus* LPS. We have been interested in examining the responsiveness of these mice to the LPS of *Pseudomonas aeruginosa*, which has a lipid A structure that more closely resembles that of the enterobacteriaceae. The lipid A portion of the LPS has been shown to be responsible for most of the biologic activities of this molecule (12, 13). A comparison between LPS preparations with similar lipid A moieties would then allow a more precise determination of what structural properties of the lipid A portion of the LPS may account for the nonresponsiveness of the C3H/HeJ mouse strain to the biologic effects of endotoxin.

P. aeruginosa is a Gram-negative rod that causes infections in immunocompromised hosts that are often fatal (14, 15). Numerous workers have thought that this endotoxin from *P. aeruginosa* is structurally and behaviorally similar to enterobacterial LPS (16–18). Although Pier *et al.* (19) and Drewry *et al.* (20) have found differences in the lipid components of *P. aeruginosa* LPS from those seen in *Escherichia coli* and *Salmonella typhimurium* LPS (16) there are some striking similarities in the ratios of hydroxy fatty acids to straight chain fatty acids, and the linkages of these 2 kinds of fatty acids to the glucosamine disaccharide backbone. In addition, the glucosamine backbone of *P. aeruginosa* has been shown to have a β 1,6 linkage like that of the enterobacteriaceae (20), and to be phosphorylated in a similar manner. We therefore have investigated the biologic responses of C3H/HeJ mice to *P. aeruginosa* LPS and determined the nature of the fatty acid linkages in the lipid A portion of this LPS. We have found that minor structural differences between *P. aeruginosa* and enterobacterial LPS have a profound impact on the ability of C3H/HeJ mice to respond to the biologic effects of these 2 endotoxins.

MATERIALS AND METHODS

Animals. Normal 8- to 40-wk-old C3H/HeJ mice were obtained from either Jackson Laboratories, Bar Harbor, ME, or the Animal Resources Center, Harvard School of Public Health. The older mice were only used in mitogenicity assays. Six- to 8-wk-old C3H/ANF and BALB/c mice were obtained from Cumberland View Farms, Clinton, TN.

Materials. LPS from *P. aeruginosa* was obtained by 2 methods from 4L of trypticase soy broth cultures grown for 48 hr at 37°C with immunotype 1 *P. aeruginosa*. The first involved phenol-water extraction of the pelleted cells by the method of Westphal and Jann (21), followed by chromatography of the ultracentrifuge pellet on a Sepharose CL 4B column, 2.6 x 100 cm, recovery, and ultrafiltration concentration of the void volume, then a final cold phenol extraction, separation of the phenol and water phases, recovery, dialysis, and lyophilization of the aqueous phase. The 2nd method involved concentration of the cultural supernatant to 250 ml on Amicon (Amicon Corp., Lexington, MA) ultrafiltration membranes (XM 100), removal of cellular debris by centrifugation at 20,000 rpm for 30 min, and pelleting out the LPS in the ultracentrifuge, resuspending the pellet in distilled water, and recovery by a second ultracentrifugation. This material was dissolved in water, extracted with cold phenol for 48 hr, the aqueous layer was then recovered, dialyzed against distilled water, and then applied to the Sepharose CL 4B column. The resultant void volume was treated as above by ultrafiltration concentrations, phenol reextraction, recovery, dialysis, and lyophilization. The LPS from the phenol-water extracted cells was desig-

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[1] This work was supported by Contract DAMD 17-70-C-9050 from the United States Army Medical Research and Development Command.
[2] Please address reprint requests to: Dr. Gerald B. Pier, Channing Laboratory, 180 Longwood Ave., Boston, MA 02115.
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nated PW-LPS,[4] and the LPS from the concentrated cultural supernatant was designated CCS-LPS. Control phenol-water LPS from *E. coli* 026:B6 was obtained from Difco Laboratories, Detroit, MI., and control *S. typhimurium* Rc mutant LPS was obtained from Dr. J. C. Sadoff, Walter Reed Army Institute of Research. High m.w. polysaccharide (PS) from immunotype 1 *P. aeruginosa*, which reacts as an immunologic identity to the "O" side chain of immunotype 1 LPS, was used in the splenic plaque-forming cell (PFC) assay and prepared as previously described (19). Isolated lipid A from *P. aeruginosa* LPS was obtained by hydrolysis of the LPS in 1% acetic acid at 90°C for 6 hr to cleave and precipitate the lipid A portion. After recovery by centrifugation the lipid A was solubilized with triethylamine and complexed to bovine serum albumin (BSA) as described (22).

Chemical analysis. Determinations of the biochemical components, monosaccharide, and lipid composition of the LPS antigens employed were done as follows: Protein was determined by the method of Lowry *et al.* (23), nucleic acid was determined by the absorbance at 254 nm of the antigens with yeast RNA as a standard (not corrected for LPS end absorption effects), monosaccharide and lipid components were determined quantitatively and qualitatively by gas liquid chromatography as described (19). 2-keto-3-deoxy octulosonic acid (KDO) was determined by the thiobarbituric acid assay (24) using purified KDO as standard (Sigma Chemical Co., St. Louis, MO), and heptose was determined by the cysteine-sulfuric acid method (24) using L-glycero-D-mannoheptose as standard, which was kindly supplied by Dr. B. Cox, National Bureau of Standards, Rockville, MD. Linkages of the fatty acids to the lipid A component of the *P. aeruginosa* LPS were determined by the methods of Rietschel *et al.* (16) and Drewry *et al.* (20), except that BF_3 methanol was used to methylate the fatty acids released by alkaline sodium methoxy treatment after the determination of the fatty acids that were released and methylated by the sodium methoxy treatment itself.

Immunogenicity studies. Spleens from mice injected with LPS were tested for PFC by a slide modification of the splenic PFC assay as described by Jerne and Nordin (25), or by the method of Cunningham and Szenberg (26). Sheep red blood cells (SRBC) were sensitized with *P. aeruginosa* PS with chromium chloride, employing 0.5 ml of packed cells, 1.0 mg of PS in 1.0 ml of 0.15 M sodium chloride, and 1.0 ml of a 0.07% solution of chromium chloride. The mixture was allowed to stand for 5 min, after which the sensitized cells were washed 3 times with saline and used at a final concentration of 10%. SRBC were sensitized with the *E. coli* 026 LPS by the method of Rudbach (27).

Assay of antibodies. Determinations of serum antibody levels to *P. aeruginosa* PS after immunization with LPS were done by using a radioactive antigen-binding assay (RABA) as described by Farr (28). Intrinsically labeled ^{14}C PS was employed as antigen[5] (specific activity 0.52 cpm/ng) at a concentration of 6000 ng/ml. Fifty microliters of antigen and 100 μl of serum were reacted for 2 hr at 37°C, then overnight at 4°C. Preparation of material for counting was done as described by Farr (28). Quantitation of the immune response measured in the RABA was done by determining antibody levels in the serum of 3 rabbits and from 2 pooled mouse sera by quantitative precipitins. Plotting of the percentage binding of these sera in the RABA *versus* the $\log_{10}$ antibody concentration and analysis by linear regression calculations yielded a coefficient of correlation (r^2) of 0.97, and the formula for calculating the serum antibody concentration. Determinations of serum antibody levels to the *E. coli* LPS were done by passive hemagglutination as described by Rudbach (27).

Mitogenicity. Cultures of 10^6 viable spleen cells were placed in round bottom microtiter wells of 7-mm diameter (Linbro Laboratories, Hamden, CT) with a total volume of 200 μl. The medium was RPMI 1640 with 10% fetal calf serum, 2 mM L-glutamine, penicillin, and streptomycin. LPS at the indicated concentration was added at the time of culture initiation. Each culture was established in duplicate and allowed to incubate for 3 days in a humidified 5% CO_2 atmosphere at 37°C. Each well received 0.2 μCi of tritiated thymidine (6.7 Ci/mmol, New England Nuclear, Boston, MA) 18 hr before cells were harvested in a multiple sample harvester (Flow Laboratories, Rockville, MD). The trichloroacetic acid-precipitable radioactivity was measured in a Beckman liquid scintillation counter (Beckman Laboratories, Palo Alto, CA). Mitogenicity studies using polymixin B sulfate (Pfizer Laboratories, New York) were performed by adding an equal weight of the antibiotic and mitogen together in the tissue culture well.

Cellular selection for mitogenesis. Treatment of murine splenocytes with antisera to the Thy 1.2 and immunoglobulin (Ig) determinants were performed as follows. Anti-Thy 1.2 alloantisera (Accurate Chemical Co., Hicksville, NY) were diluted 1/20 in Hanks' balanced salts solution (HBSS) and harvested, washed splenocytes were resuspended in this solution at a concentration of 10^7 cells/ml. The cell suspension was incubated at 4°C for 60 min, the cells were recovered by centrifugation and resuspended in a 1/20 dilution of rabbit Lo-tox complement (C), (Accurate Chemical Co.) in HBSS, and incubated at 37°C for 60 min. The cells were then recovered by centrifugation and resuspended in a volume of the RPMI 1640 tissue culture medium equal to the original volume giving 10^7 cells/ml. Treatment of Ig-bearing splenocytes was done in a similar manner, except that rabbit anti-mouse-Ig (Accurate Chemical Co.) was employed in the presence of 0.01% azide. Cell controls for these studies were cells treated with buffer and C alone. The effectiveness of the anti-Thy 1.2 serum treatment was measured by the reduction in mitogenesis induced by the T cell mitogen, phytohemagglutinin (PHA), and the effectiveness of the anti-Ig serum treatment was assessed by the reduction in LPS-induced mitogenesis.

Lethality. The lethal effects of the LPS preparations were assayed in mice by the i.p. injection of graded doses of LPS in 0.5 ml saline. Endotoxemia was evaluated by the appearance of the following symptoms: closed, crusted over eyes, rigors, abnormal appearance of fur, and diarrhea.

Short term refractivity to LPS lethality. C3H/ANF mice were primed with sublethal doses of the *P. aeruginosa* and *E. coli* LPS and challenged 30 hr later with a previously determined lethal dose of these antigens in order to assess short-term refractivity to the lethal effects of endotoxin after priming with sublethal doses.

RESULTS

Chemical composition. Table I lists the various chemical components found in the *P. aeruginosa* and *E. coli* LPS used. The 2 *P. aeruginosa* specimens were essentially identical in terms of their contamination with protein and nucleic acids, whereas the *E. coli* LPS contained a high level of protein contamination but a reduced level of nucleic acid contamination. As previously reported (18), *P. aeruginosa* LPS has a high carbohydrate to lipid weight ratio whereas the *E. coli* LPS had nearly a 1:1 weight ratio of carbohydrate to lipid. These ratio values were based on the weight percentage of totally identified lipid and carbohydrate compounds in the LPS preparations, excluding ribose and deoxyribose.

Table II shows the monosaccharide composition of the *P. aeruginosa* LPS used. Both preparations had similar KDO and heptose contents, with the qualitative and quantitative composition of the remaining monosaccharides being very similar. This confirmed that the 2 isolation procedures used to obtain LPS from *P. aeruginosa* yielded essentially identical materials.

Table III lists the lipid components found in the 2 *P. aeruginosa* and the *E. coli* LPS employed. *P. aeruginosa* LPS showed a qualitative difference in lipid composition from that of *E. coli* LPS, but both showed a high molar ratio of total hydroxy fatty acids relative to the molar ratio of total straight chain fatty acids. We again found unsaturated C 16 and C 18 fatty acids in the *P*

TABLE I

Comparison of the chemical components in the LPS antigen preparations used

Component[a]	*P. aeruginosa* LPS		*E. coli* LPS
	PW-LPS	CCS-LPS	
Carbohydrate	76.2	81.4	[illegible]
Lipid	8.3	9.5	3[illegible]
Protein	0.8	0.6	21.7
Nucleic acid	6.6	5.4	1.5
Total percentage identified	91.9	97.5	99.7

[a] See *Materials and Methods* for procedures used.

[b] PW-LPS, phenol water extracted *P. aeruginosa* LPS. CCS-LPS, concentrated cultural supernatant LPS.

[c] Represents weight percentage determined.

TABLE II

Monosaccharide composition of the P. aeruginosa LPS antigens

	Monosaccharide							
	rham[a]	man	glu	KDO	gluam	galam	hep	2,6-dideoxy gal
PW-LPS	1.77[b]	0.09	2.11	0.20	0.29	0.29	0.20	+ +
CCS-LPS	1.86	0.13	2.14	0.19	0.26	0.31	0.24	+ +

[a] rham, rhamnose; man, mannose; glu, glucose; KDO, 2-keto-3-deoxyoctonoate; gluam, 2-acetamido-2-deoxyglucose; galam, 2-acetamido-2-deoxygalactose; hep, L-glycero-*d*-manno heptose; 2,6-dideoxy gal, 2-acetamido,2,6-dideoxygalactose.

[b] Micromoles monosaccharide per milligram dry weight.

[c] Not quantifiable due to unavailability of standard.

[4] Abbreviations used in this paper: PW-LPS, LPS from phenol-water extracted cells; PS, polysaccharide; CCS-LPS, LPS from concentrated culture of supernatant; RABA, radioactive antigen-binding assay; KDO, 2-keto-3-deoxy octulosonic acid; EDTA, ethylenediaminetetraacetic acid; HBSS, Hanks' balanced salts solution; LPA, lipid A protein.

[5] Pier, Gerald B. 1981. Cross protective efficacy of high molecular weight polysaccharides from immunotypes 1 and 2 *Pseudomonas aeruginosa*. Submitted for publication.

aeruginosa LPS as we have previously reported (19), whereas other workers (17, 20) did not find these fatty acids.

The linkages of the fatty acids to the lipid A portion of the *P. aeruginosa* and *E. coli* LPS were determined by alkaline hydroxyaminolysis and alkaline methanolysis (15, 20). Our results are depicted in Figure 1 and Table IV. The linkages determined for the fatty acids in the *P. aeruginosa* LPS are consistent with the results of Drewry *et al.* (20) showing that unsubstituted 3 hydroxydodecanoic acid (3:OH C 12) is linked to the amide residues on the glucosamine disaccharide backbone (Ref. point 1, Fig. 1), whereas dodecanoic acid (C 12), 3 hydroxydecanoic acid (3:OH C 10), and a trace amount of 3 hydroxydodecanoic acid (3:OH C 12) are directly ester linked to the free hydroxyl groups of the glucosamine disaccharide (Ref. points 2 and 3, Fig. 1). 2 Hydroxydodecanoic acid (2:OH C 12), hexadecanoic acid (C 16), hexadecenoic acid (C 16:1), octadecanoic acid (C 18), and octadecenoic acid (C 18:1) are likely ester linked to the 3 hydroxydecanoic acid (3:OH C10) that is directly ester linked to the glucosamine disaccharide backbone (Ref. point 4, Fig. 1).

The fatty acid linkages in the *E. coli* LPS were found to be similar to those reported by Rietschel *et al.* (16) for *S. typhimurium* LPS, showing unsubstituted 3 hydroxytetradecanoic acid (3:OH C 14, Ref. point 1', Fig. 1) linked to the amide residues of the glucosamine backbone, while ester linked to the free hydroxyl groups were dodecanoic acid (C 12), hexadecanoic acid (C 16), and 3 hydroxytetradecanoic acid (3:OH C 14, Ref. points 2' and 3', Fig. 1), and linked to the 3 hydroxyl group of the ester linked 3 hydroxytetradecanoic acid (3:OH C 14) was tetradecanoic acid (C 14, Ref. point 4', Fig. 1). Thus, the major differences seen between the *P. aerguinosa* and *E. coli* lipid A structures were in the length of the carbon chains of the substituent fatty acids, and the nature of the fatty acid substituent on the hydroxy fatty acid directly ester linked to the glucosamine disaccharide backbone (Ref. points 4 and 4', Fig. 1).

Immunogenicity. Figure 2 shows the dose-response curve of 3 mouse strains in the splenic PFC assay at day 5 to immunization with *P. aeruginosa* LPS. There is little difference in the response to this antigen between the C3H/HeJ and C3H/ANF mouse strains. BALB/c mice are slightly more responsive to lower doses of *P. aeruginosa* LPS than are both the C3H strains, but less responsive to higher doses of LPS. Measurements of the serum antibody levels from these mice (Table V) confirms no apparent differences in the responses of the 2 C3H mouse strains to *P. aeruginosa* LPS.

Figure 3 shows the dose-response curve of the 2 C3H mouse strains to the *E. coli* 026 LPS in the splenic PFC assay at 5 days after immunization. These data duplicate the results of Watson and Riblet (2) showing that the responder C3H/ANF mice make a significant PFC response over a wide range of LPS doses, whereas the nonresponder C3H/HeJ strain make a lower response over a narrow dosage range. Serum titers from these mice in a hemagglutination assay are listed in Table VI, and again parallel the findings of Watson and Riblet (2).

Mitogenicity. Figure 4 depicts the net incorporation of tritiated thymidine, expressed as the difference in cpm of ^3H incorporated into LPS stimulated cultures from unstimulated controls, (background counts were 4890 cpm in C3H/HeJ cultures, and 4732 in C3H/ANF cultures) in spleen cell cultures taken from C3H/ANF and C3H/HeJ mice stimulated with both *P. aeruginosa* and *E. coli*

TABLE III

Lipid composition of P. aeruginosa and E. coli LPS antigens

Lipid	Antigen		
	P. aeruginosa		*E. coli* LPS
	PW-LPS	CCS-LPS	
3:OH C10[a]	0.12[b]	0.14	0
2:OH C12	0.36	0.34	0
3:OH C12	0.33	0.31	0
3:OH C14	0	0	0.69
Total molar ratio hydroxy fatty acids/total moles fatty acids found	0.81	0.79	0.69
C 12	0.06	0.08	0.05
C 14	0	0	0.16
C 16	0.05	0.04	0.10
C 16:1	0.03	0.02	0
C 18	0.01	0.03	0
C 18:1	0.04	0.04	0
Total molar ratio of straight chain fatty acids/total moles fatty acids found	0.19	0.21	0.31

[a] 3:OH C10, 3-hydroxydecanoic acid; 2:OH C12, 2-hydroxydodecanoic acid, 3 OH C12, 3-hydroxydodecanoic acid; 3 OH C14, 3-hydroxytetradecanoic acid, C 12, dodecanoic acid, C 14, tetradecanoic acid, C 16, hexadecanoic acid, C 16 1 hexadecenoic acid, C 18, octadecanoic acid, C18 1, octadecenoic acid

[b] Molar ratio of lipids per total moles identified fatty acids

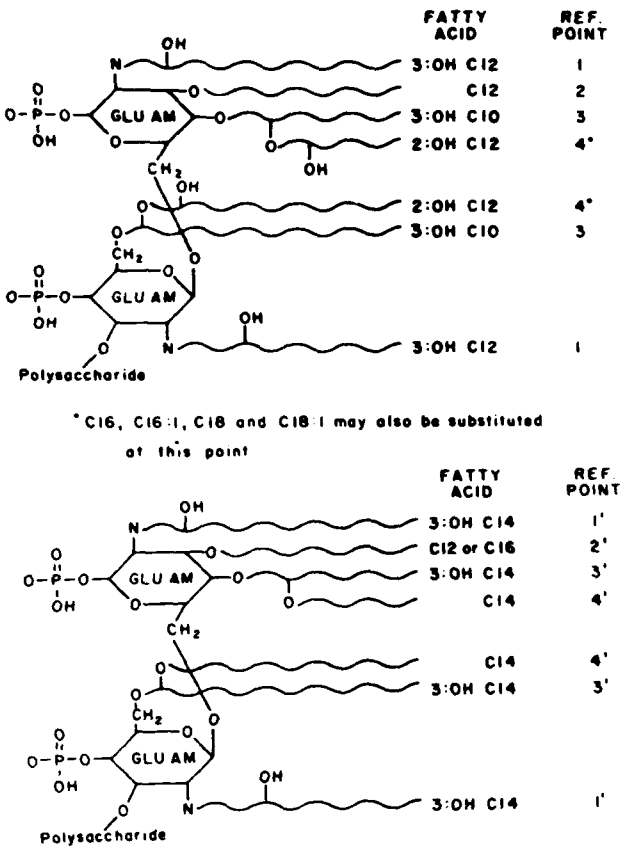

Figure 1. Structural depiction of the fatty acid substituents and their linkages to the glucoseamine disaccharide backbone in *Pseudomonas aeruginosa (upper)* and *Escherichia coli (lower)* LPS. Ref. (reference) points refer to those linkage positions between the 2 structures that indicate the points of comparison in the text. Abreviations: GLU AM, 2-amino-2-deoxy glucose; OH, hydroxyl substituent; 3:OH C-12, 3-hydroxydodecanoic acid; C12, dodecanoic acid; 3:OH C10, 3 hydroxydecanoic acid; 2:OH C12, C-hydroxydodecanoic acid; 3:OH C14, 3-hydroxytetradecanoic acid; C16, hexadecanoic acid, C16:1, hexadecenoic acid; C18, octadecanoic acid; C18:1 octadecenoic acid. Figure is drawn after method of Galanos *et al.* (39).

TABLE IV

Release of fatty acids after mild alkaline methanolysis of lipid A fractions (A) and fatty acids present after methylation with BF_3 methanol (B)[a]

Lipid	*P. aeruginosa*		*E. coli*	
	A	B	A	B
Dodecanoic acid	319[b]	337	312	347
Tetradecanoic acid			Trace	512
Hexadecanoic acid	2	3	218	201
3-Hydroxydecanoic acid	791	776		
2-Hydroxydodecanoic acid	Trace	419		
3-Hydroxydodecanoic acid	19	22		
3-Hydroxytetradecanoic acid			616	682
Minor components[c]	Trace	53		

[a] After Drewry *et al.* (18).

[b] Represents ratio of peak area found to that relative to an internal standard of methyl heptadecanoate as 1000.

[c] Minor components hexadecanoic acid (C16), hexedecenoic acid (C16:1), octadecanoic acid (C18), and octadecenoic acid (C18:1).

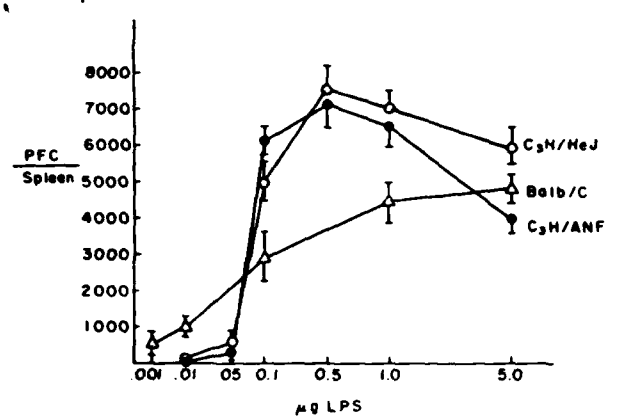

Figure 2 Dose response curve in the splenic PFC assay after immunization with LPS from *Pseudomonas aeruginosa*. PFC/spleen indicate total plaques calculated per individual mouse spleen. Points indicate average of 5 mice and bar represents ± SD.

TABLE V

Serum antibody levels of mice immunized with the PW-LPS from P. aeruginosa

Dosage LPS	Mouse Strain		
	BALB/C	C3H/ANF	C3H/HeJ
μg			
0.001	9.4[a]	ND[b]	ND
0.01	56.8	<2.0	<2.0
0.05	ND	5.5	8.4
0.10	84.2	ND	ND
0.50	62.8	31.7	48.7
1.00	43.2	44.1	63.8
5.00	62.3	26.6	28.2

[a] Represents average micrograms per milliliter of serum antibody from 5 individual mice immunized with indicated dosage of LPS 5 days postimmunization.
[b] ND, not determined.

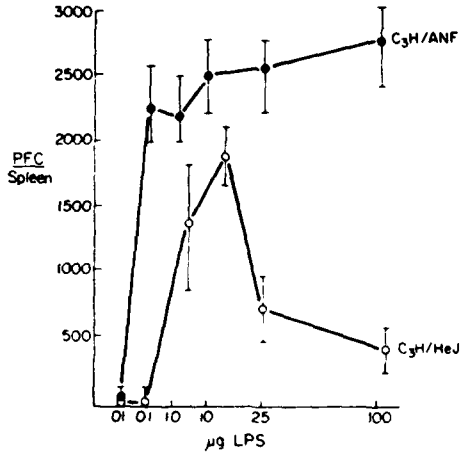

Figure 3 Dose response curve in the splenic PFC assay after immunization with *E. coli* 026 LPS. PFC/spleen indicate total plaques calculated per individual mouse spleen. Points indicate average of 5 mice and bar represents ± SD.

LPS. It can be seen that the C3H/HeJ mice respond mitogenically to *P. aeruginosa* LPS but to a lesser degree than do C3H/ANF mice. The maximal response of these 2 mouse strains occurs at closely related doses (5 to 25 μg/ml) of the *P. aeruginosa* LPS, but the maximal response of C3H/HeJ spleen cell cultures at 5.0 μg/ml is only 37.6% of the maximal C3H/ANF response at 25.0 μg/ml. At the highest dose tested of *P. aeruginosa* LPS, 100 μg/ml, C3H/ANF spleen cells are still maximally responsive whereas the C3H/HeJ response has precipitously fallen off. The response to the *E. coli* LPS is also shown in Figure 4 and indicates that the C3H/HeJ spleen cells were totally refractive to mitogenic stimulation by this LPS, whereas the C3H/ANF mice were totally responsive. For both mouse strains, doses of *E. coli* LPS equal to or greater than 100 μg/ml were highly inhibitory to the cultures. Interestingly, the dose-response curve of the C3H/ANF mice to the *E. coli* LPS was identical to that for *P. aeruginosa* LPS. Mitogenicity studies on the "O" side chain polysaccharide from *P. aeruginosa* and *E. coli* LPS indicate that these polysaccharides themselves are not mitogenic in this dosage range (manuscript in preparation). This clearly implicates the lipid A from *P. aeruginosa* LPS as being responsible for the mitogenicity observed.

To test further the hypothesis that lipid A is the mitogenically important component of *P. aeruginosa* LPS, we studied the mitogenicity of *P. aeruginosa* LPS on purified T and B cells from C3H/ANF and C3H/HeJ spleens, as well as the mitogenicity of solubilized lipid A-BSA on intact splenocytes. Table VII shows that in both mouse strains anti Thy 1.2 + C treatment had little or no effect on reducing the mitogenicity of the *P. aeruginosa* LPS when

TABLE VI

Serum antibody titer of mice immunized with E. coli LPS

Dosage LPS	Mouse Strain	
	C3H/ANF	C3H/HeJ
μg		
0	<2[a]	<2
0.1	32	4
1.0	256	16
10.0	512	8
25.0	512	16
100.0	128	4

[a] Represents reciprocal of serial 2-fold serum dilution showing positive agglutination for sensitized sheep RBC.

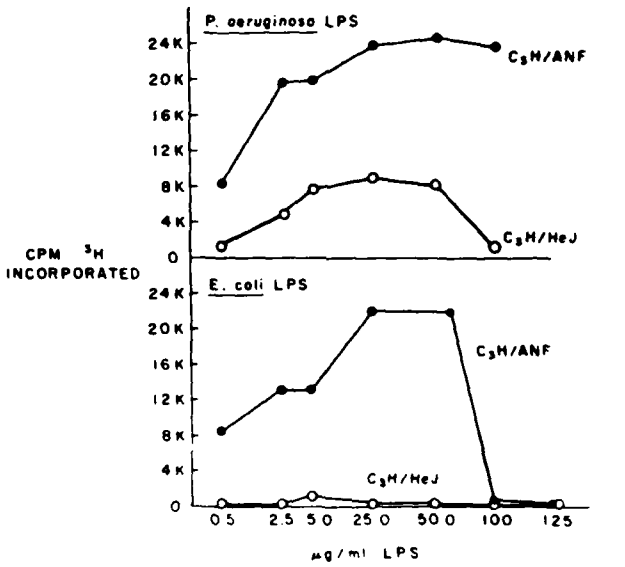

Figure 4 Seventy-two hour incorporation of tritiated thymidine (^{3}H) into spleen cell cultures from C3H, ANF and C3H/HeJ mice after stimulation by the indicated doseage of LPS. Points indicate average incorporation of ^{3}H into duplicate cultures after subtraction of the cpm of ^{3}H incorporated into unstimulated, control cultures (see text for background cpm subtracted)

TABLE VII

Mitogenic responses of C3H ANF and C3H/HeJ splenocytes to P. aeruginosa LPS (PA-LPS), E. coli LPS (EC-LPS) and PHA after treatment of cells with anti-Thy 1.2 or anti-Ig plus C

Cell Treatment	Mouse Strain					
	C3H ANF			C3H HeJ		
	PA-LPS	EC-LPS	PHA	PA-LPS	EC-LPS	PHA
C only	44.8[a]	48.0	18.8	10.7	1.2	34.2
Anti-Thy 1.2 + C	46.5	46.6	1.4	9.4	1.0	<BKD[b]
Anti-Ig + C	16.2	11.8	23.5	1.7	0.2	24.3

[a] Represents cpm × 10^3 incorporated ^{3}H thymidine minus the background counts; background values (average cpm of duplicate cultures) C only treated cells, 4118 for C3H/ANF mice, 4569 for C3H/HeJ mice; anti-Thy 1.2-treated cells, 3071 for C3H/ANF mice, 3945 for C3H/HeJ mice; anti-Ig-treated cells, 1093 for C3H/ANF mice, 1613 for C3H/HeJ mice
[b] BKD, background

compared to the mitogenicity induced in C only treated control cells. A similar effect was noted in the responder mice with the *E. coli* LPS. This treatment did reduce the PHA-induced mitogenesis by 92.7% in C3H/ANF mice and by 100% in C3H/HeJ mice. In contrast, anti-Ig + C treatment of cells reduced the *P. aeruginosa* and *E. coli* LPS-induced mitogenesis by 63.7 and 75.4%, respectively, in responder C3H/ANF mice, and the *P. aeruginosa* LPS-induced mitogenesis by 71.3% in nonresponder C3H/HeJ mice. Reduction of PHA-induced mitogenesis in anti-Ig + C-treated cells was negligible or increased. Thus, the *P. aeruginosa* LPS preparation was a mitogen for Ig but not Thy 1.2-bearing cells, supporting the view that this LPS is a polyclonal B cell mitogen in both responder and nonresponder C3H mice. This property has been shown to be induced by the lipid A portion of the LPS (12).

Next we examined the ability of polymixin B to inhibit the spleen cell mitogenesis induced by intact LPS and solubilized lipid A-BSA preparations in C3H/ANF and C3H/HeJ mice. This antibiotic has been shown to block LPS-induced lymphocyte mitogenesis (29). Table VIII shows that polymixin B reduced the mitogenesis induced by the *P. aeruginosa* and *E. coli* intact LPS in C3H/ANF mice by 86.8 and 94.0%, respectively, when compared to the mitogenesis induced in the absence of the antibiotic. In C3H/HeJ mice, the *P. aeruginosa* LPS-induced mitogenesis was reduced by 83.1% in the presence of polymixin B, whereas the low level of mitogenesis seen in this experiment induced by *E. coli* LPS was unaffected by the antibiotic. Solubilized lipid A-BSA from *P. aeruginosa* was mitogenic for both the C3H/ANF and C3H/HeJ spleen cells to a degree comparable to that of the intact LPS, and this reactivity was also inhibitable by polymixin B.

Toxicity and lethality for mice. Figure 5 shows the dose-response curve of C3H/ANF and C3H/HeJ mice to graded doses of the *P. aeruginosa* endotoxin. The grading of endotoxemia was done by an observer every 8 hr who was unaware of the mouse strain or doseage of endotoxin used. TheC3H/HeJ mice showed susceptibility to the endotoxin poisoning effects of *P. aeruginosa* LPS, but it required higher doses to elicit these effects than in the C3H/ANF mice and the toxicity was of a lesser intensity and shorter duration in C3H/HeJ. C3H/ANF mice were killed uniformly by a

TABLE VIII

Inhibition of spleen cell mitogenesis by polymixin B induced by P. aeruginosa and E. coli LPS and solubilized lipid A in C3H/ANF and C3H/HeJ mice

Mitogen	Mouse Strain					
	C3H/ANF			C3H/HeJ		
	-PB[a]	+PB	% Reduction	-PB	+PB	% Reduction
PA-LPS[b]	44.8[c]	5.9	86.8	10.7	1.8	83.1
EC-LPS	48.0	2.9	94.0	1.2	1.1	8.3
Lipid A-BSA[d]	32.9	1.8	94.6	10.9	0.9	91.8

[a] PB, polymixin B sulfate

[b] PA-LPS, *P. aeruginosa* LPS; EC-LPS, *E. coli* LPS

[c] cpm × 10^3 of incorporated ^{3}H thymidine minus background (see Table VII for background to C only treated cells)

[d] Isolated lipid A from *P. aeruginosa* solubilized with triethylamine and complexed to BSA; BSA alone was not mitogenic

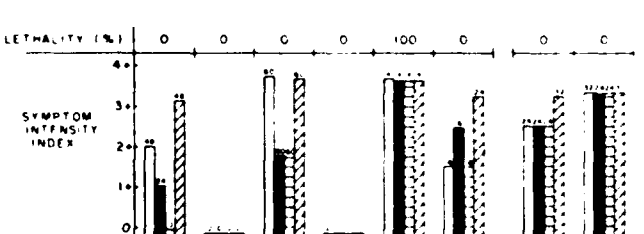

Figure 5. Endotoxin poisoning dose-response curve, as measured by the symptom intensity index, of C3H/ANF and C3H/HeJ mice, after injection of the indicated dose of *Pseudomonas aeruginosa* LPS. Numbers above the bars refer to the duration, in hours, of the symptom from the time a 1+ or greater reaction was noted to the time that the reaction had subsided. The crosses atop the bars for the C3H/ANF mice given 10.0 mg/kg was due to the fact that these mice all died by 24 hr. The percentage lethality is shown on the line across the top. □, diarrhea; ■, rigors; ▤, ruffled fur; ▨, closed, crusted over eyes.

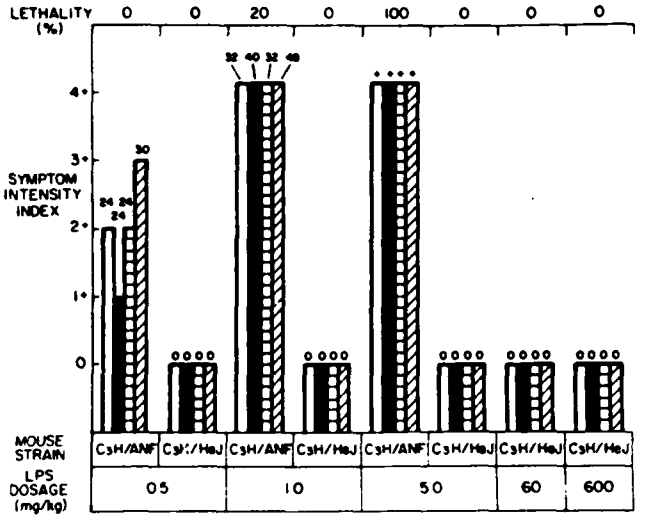

Figure 6. Endotoxin poisoning dose-response curve, as measured by the symptom intensity index, of C3H/ANF and C3H/HeJ mice, after injection of the indicated dose of *E. coli* LPS. Numbers above the bars refer to the duration, in hours, of the symptom from the time a 1+ or greater reaction was noted to the time the symptom subsided. The crosses atop the bars for the C3H/ANF mice given 5.0 mg/kg indicate that these mice all died by 30 hr. The percentage lethality is shown on the line across the top. □, diarrhea; ■, rigors; ▤, ruffled fur; ▨, closed crusted over eyes.

TABLE IX

Results of short-term refractivity to a lethal dose of LPS after a sublethal priming dose 30 hr previously with the indicated LPS in C3H/ANF mice

Challenging LPS (Dosage μg)	Priming Material		
	E. coli LPS (50 μg)	*P. aeruginosa* PW-LPS (50 μg)	Saline
E. coli (125)	5/5[a]	0/5	0/5
S. typhimurium (125)	5/5	0/5	0/5
P. aeruginosa PW-LPS (250)	0/5	0/5	0/5

[a] Represents number of mice over total injected surviving at 96 hr after the challenge dose.

dose of *P. aeruginosa* LPS of 10 mg/kg, but the C3H/HeJ mice survived doses of *P. aeruginosa* LPS as high as 600 mg/kg. *E. coli* LPS was both toxic and lethal for C3H/ANF mice, with 100% mortality seen at a dosage of 5.0 mg/kg (Fig. 6). *E. coli* LPS was without either toxic or lethal effects in C3H/HeJ mice at doses as high as 600 mg/kg (about 12 mg/mouse).

Short-term refractivity to LPS lethality. Greisman *et al.* (30) have shown that LPS-primed rabbits are refractive to the pyrogenic effect of a 2nd dose of endotoxin within the first 24 to 48 hr. This refractivity is nonspecific for endotoxins from different species. We were interested in seeing if a similar phenomenon, as measured by refractivity to lethality, was operative in mice, and if this refractivity was specific for enterobacterial LPS. We therefore primed C3H/ANF mice with sublethal doses (1.0 mg/kg) of *P. aeruginosa* and *E. coli* LPS' and challenged them 30 hr later with one 100% lethal dose of either the homologous priming endotoxin, the heterologous *E. coli*, or *P. aeruginosa* endotoxin, or a heterologous *S. typhimurium* endotoxin. Table IX shows that whereas mice primed with *E. coli* LPS were refractive to the lethal effects of both the homologous *E. coli* LPS and the heterologous *S. typhimurium* LPS, they were not protected against challenge with the *P. aeruginosa* LPS. Mice primed with the *P. aeruginosa* LPS were not refractive to the lethal effects of any of the challenging endotoxins. This showed that no antibody to the *P. aeruginosa* LPS had developed, since this antibody is protective against endotoxemia caused by *P. aeruginosa* LPS (unpublished observation).

DISCUSSION

These data clearly indicate that the refractivity of C3H/HeJ mice to the biologic effects of enterobacterial endotoxin is not seen with

the LPS from *P. aeruginosa*, which proved to be immunogenic, mitogenic, and toxic in this "unresponsive" substrain of C3H mouse. These results suggest that the inability of this mouse substrain to respond to LPS may be limited to the endotoxin from members of the family enterobacteriaceae. The results of Spellman and Reed (8) and Moreno and Berman (9) regarding the biologic effects of *B. abortus* LPS in C3H/HeJ mice also support this conclusion, and recently Moreno *et al.* (22) have shown that this mitogenesis is due to the lipid A portion of the *B. abortus* LPS.

Structural analysis of the lipid linkages to the lipid A component of *P. aeruginosa* LPS showed that *P. aeruginosa*'s LPS had qualitatively different fatty acids from the *E. coli* LPS, but similarities were noted in terms of the ratio of hydroxy fatty acids to straight chain fatty acids, and the linkages of these 2 different types of fatty acids to the glucosamine backbone. Figure 1 diagrams these results, and points out those linkage positions where the fatty acid substituents are similar and different. Information available on the structure of the LPS of *B. abortus* indicates that its fatty acid substituents differ considerably more from the enterobacterial LPS than do the fatty acid substituents of *P. aeruginosa* LPS (10, 11), although there is some heterogeneity in the fatty acid substituents found in enterobacterial LPS (11). Even though the *P. aeruginosa* LPS and the *E. coli* LPS are structurally similar in their lipid A portion, they differ substantially in their biologic effects in C3H/HeJ mice. These biologic activities of LPS must therefore depend critically on those structural features that distinguish the enterobacterial LPS from that of *P. aeruginosa*.

The chemical components present in the 2 *P. aeruginosa* LPS preparations used indicate a very low level of protein contamination. Lipid A protein (LAP) present in butanol-extracted enterobacterial LPS (31), and outer membrane proteins from *P. aeruginosa* strain PAO (32), have both been shown to cause mitogenesis in C3H/HeJ lymphocytes. It is, however, highly unlikely that the mitogenic responses we observed in spleen cell cultures stimulated with *P. aeruginosa* LPS were due to contamination of our LPS preparations with such proteins. The minimum dose of *P. aeruginosa* outer membrane proteins required to produce mitogenesis far exceeds the total protein contamination present in our mitogenic doses of *P. aeruginosa* LPS (32). Furthermore, the *P. aeruginosa* LPS preparations used were phenol extracted numerous times, a procedure known to eliminate LAP and mitogens from butanol-extracted enterobacterial LPS (33). Thirdly, the *P. aeruginosa* LPS-induced splenocyte mitogenesis in both C3H/ANF and C3H/HeJ mice was highly inhibited by the antibiotic polymixin B, which binds specifically to the lipid A portion of LPS molecules and interferes with LPS-induced biologic phenomena (34). Additionally, even though Skidmore *et al.* (33) reported that Westphal phenol-water extracted *E. coli* (111:B4 LPS was mitogenic in C3H/HeJ mice, this activity was only 13.1% of the activity seen in responder C3H/St mice, whereas our *P. aeruginosa* LPS had activity in C3H/HeJ mouse cells equal to 37.6% of the response seen in C3H/ANF mouse cells. These workers also reported that C3H/HeJ mice had a lower mitogenic activity to all LPS' that were capable of inducing proliferation in this strain when compared to the activity of the corresponding LPS preparation in C3H/St mice. Finally, the presence of 21.7% protein in our commercial *E. coli* LPS preparation did not make this preparation mitogenically active in the C3H/HeJ mice, indicating that protein contamination of LPS is in itself not a measure of the ability of enterobacterial endotoxin's to be biologically active in nonresponding mouse strains.

The equivalent immunogenicity of *P. aeruginosa* LPS in C3H/HeJ mice and C3H/ANF mice contrasts with the different immune responses to enterobacterial LPS observed by others between C3H/HeJ mice and other C3H mouse substrains (2, 6). This infers that structural differences between the lipid A portion of the *P. aeruginosa* and *E. coli* LPS are sufficient to make the *P. aeruginosa* LPS a good immunogen in C3H/HeJ mice. Our data (Fig. 3) are consistent with the report of Watson and Riblet (2) showing a significant difference between the PFC responses of responder and nonresponder mice to a wide range of doses of *E. coli* LPS. On the other hand, Skidmore *et al.* (6) showed that C3H/HeJ mice made an equivalent day-5 primary PFC response to high doses (25 μg) of *E. coli* 0113 and K235 LPS when compared to the response of C3H/St mice. However, the C3H/St mice showed a prolonged PFC response whereas the C3H/HeJ response fell off rapidly. In our hands, the PFC response to *P. aeruginosa* LPS fell off rapidly after day 5 in both strains, and this decline held over a wide range of doses (data not shown). The explanation for this is not clear but suggests that the prolonged synthesis of antibody is not due to mitogenicity *per se*.

In another study we found that acid hydrolyzed PS side chains from *P. aeruginosa* LPS, high m.w. PS (19), and alkali detoxified LPS are not immunogenic at doses of less than 1 μg (manuscript in preparation), whereas intact LPS is. None of these materials contains active lipid A. Thus, although Von Eschen and Rudbach (35) suggest that the lipid A portion of enterobacterial LPS is not essential for murine primary immune responses to LPS, our data indicate the lipid A is essential for primary immune responses to *P. aeruginosa* LPS in the nanogram dosage range reported here. The highly immunogenic nature of the very low doses of *P. aeruginosa* LPS in another mouse strain, BALB/c, shows that this low dose immunogenicity extends across H-2 types.

Spleen cell proliferation induced *in vitro* by *P. aeruginosa* LPS is reduced in C3H/HeJ mice when compared to the magnitude of the proliferative response of C3H/ANF spleen cells. When contrasted to the complete unresponsiveness of C3H/HeJ spleen cells to the *E. coli* LPS, however, it is clear that the *P. aeruginosa* LPS is mitogenic for this mouse strain's spleen cells. This observation suggests that the structural similarities between the 2 LPS antigens could account for the relatively reduced mitogenic response to *P. aeruginosa* LPS in C3H/HeJ mice, whereas the structural differences account for the degree of responsiveness that does exist. The dose-response curve to *P. aeruginosa* LPS was similar in C3H/HeJ and C3H/ANF mice, with as little as 0.5 μg/ml of *P. aeruginosa* LPS giving an obvious mitogenic response with a maximum response in the 5 to 25 μg/ml range. Furthermore, isolated and solubilized lipid A from the *P. aeruginosa* LPS was also mitogenic for C3H/HeJ splenocytes, confirming that it was the lipid A portion of the LPS that was responsible for the proliferative responses seen. Chen *et al.* (32) reported that the LPS from *P. aeruginosa* strain PAO is not mitogenic for C3H/HeJ spleen cells. Because their report did not include information on the chemical composition of their LPS, and because they did not show their LPS preparation to be mitogenic in a responding C3H substrain of mice, it is difficult to determine why their observations differs with ours.

Morrison and Curry (36) extracted LPS from *P. aeruginosa* with saline, ethylenediaminetetraacetic acid (EDTA) and phenol and reported that whereas the phenol extract was mitogenic for responder C3H/St mice, and this response was inhibitable by polymixin B, their phenol LPS preparation was not mitogenic for C3H/HeJ splenocytes. However, their EDTA extracted *P. aeruginosa* LPS was mitogenic for the responder C3H/St cells, and also appeared to have mitogenic activity toward the C3H/HeJ splenocytes comparable to what we obtained here. They found in C3H/HeJ splenocytes a 37.8% inhibition of their EDTA-LPS mitogenesis by polymixin B, less than half the inhibition we found, but nonetheless supports the view that *P. aeruginosa* LPS is capable of inducing mitogenesis in C3H/HeJ spleen cells due to its lipid A. Furthermore, it should be pointed out that their phenol-extracted LPS came from the pellet of their EDTA-extracted organisms. It is well documented (37) that some strains of *P. aeruginosa* are killed by EDTA, and this killing is due to the release of the LPS from the cell wall. Similar extractions of other Gram-negative bacteria are not bactericidal (37), indicating that the products remaining in the cell wall of EDTA-sensitive strains of *P. aeruginosa* after EDTA extraction may be highly altered or disrupted (37). If Morrison and Curry (36) had used an EDTA-sensitive strain of *P. aeruginosa* to isolate their LPS from, then their phenol-extracted LPS may have been altered. Their results with EDTA-extracted LPS only conflict with ours in the degree to which polymixin B inhibited the *P. aeruginosa* LPS-induced mitogenesis in C3H/HeJ splenocytes.

A further possible explanation for the lack of mitogenesis shown by their phenol extracted *P. aeruginosa* LPS in C3H/HeJ cells may be attributable to the low level of LPS, and consequent high level of impurities, in this preparation. These impurities could interfere with LPS-induced mitogenesis. Their reported value of 10.7 nmols KDO/mg of LPS is only 5.5% of the average value of 194 nmols

KDO/mg LPS we found for the preparations of *P. aeruginosa* LPS used here. This figure is very consistent among the LPS from all of the Fisher types of *P. aeruginosa* that we work with (unpublished observation). Since their report indicated that saline-extracted *P. aeruginosa* LPS had only moderate mitogenic activity that was not polymixin B inhibitable in responder C3H/St cells, yet contained detectable quantities of LPS, as measured by the presence of KDO, it is reasonable to speculate that impure *P. aeruginosa* LPS preparations contain substances inhibitory to *P. aeruginosa*-LPS induced mitogenesis. They indicated further in their report that they performed their mitogenesis assays at numerous doses such that if the problem were solely low levels of LPS in their preparations, and not inhibitory substances, then their saline-extracted LPS should have been as mitogenic, albeit at higher doses, as their phenol-extracted LPS in responder mice. These inhibitory substances may not have been present in high enough concentration to inhibit the phenol-LPS mitogenesis in responder mice, but may have been present at high enough doses to interfere with the lessened mitogenic response of C3H/HeJ splenocytes.

The results of the spleen cell mitogenesis assay are not the only data we presented regarding positive biologic responses of C3H/HeJ mice to *P. aeruginosa* LPS. In addition to defining the chemial composition of our *P. aeruginosa* LPS preparation to ensure that its composition was similar to that previously described for *P. aeruginosa* LPS (17, 20), we also demonstrated that it produced in C3H/HeJ mice an immune response characteristic of other mouse strain responses to *P. aeruginosa* LPS, as well as producing symptoms of intoxication in C3H/HeJ mice indicative of endotoxin poisoning.

In a pattern similar to their partial sensitivity to the mitogenic effects of *P. aeruginosa* LPS, the response of C3H/HeJ mice to the toxic effects of this LPS was also muted, although clearly present. This may relate to both the similarity in structure of the 2 endotoxins as well as to their relative abilities to induce the cascade of biologic phenomena associated with toxicity and lethality. Neither *P. aeruginosa* or *E. coli* LPS were lethal in C3H/HeJ mice at doses as high as 600 mg/kg. However, endotoxin-poisoning symptoms did appear in C3H/HeJ mice after administration of *P. aeruginosa* LPS but not *E. coli* LPS. Thus, crusted over eyes, diarrhea, rigors, and ruffled fur were seen in C3H/HeJ mice given *P. aeruginosa* LPS at doses as low as 10 mg/kg, but the duration of these symptoms were shorter and their severity less than in the C3H/ANF mice. We were unable to detect any toxic or lethal effects of our *E. coli* LPS preparation in C3H/HeJ mice, although preparations of enterobacterial LPS have been shown to be toxic at high doses to these mice (1).

A final test of the degree to which the *P. aeruginosa* and enterobacterial LPS differ, and how these differences relate to the biologic effects of their endotoxins, was seen in the experiments testing the cross-refractivity to lethal challenge with LPS after a sublethal priming dose. Nonspecific refractivity to endotoxin death after a single sublethal priming dose of LPS in C3H/ANF mice was demonstrated with the *E. coli* LPS when the secondary challenging LPS was either the homologous *E. coli* LPS or a heterologous enterobacterial LPS from *S. typhimurium*. However, no refractivity to challenge with a lethal dose of *P. aeruginosa* LPS was seen with *E. coli* LPS priming, nor could *P. aeruginosa* LPS priming prevent death from a subsequent lethal dose of either *P. aeruginosa* or *E. coli* LPS. Skarnes (38) has shown that the circulating plasma of rabbits after sublethal endotoxin administration has a decreased level of ionized calcium and an increased level of organo-phosphate esterases, both of which are associated with an increased detoxification capacity of the plasma. We have demonstrated here in C3H/ANF mice an early refractivity to enterobacterial LPS lethality after a single sublethal priming dose, and shown that the refractivity extends to LPS from different enterobacterial species, but not to other Gram-negative endotoxins, such as that from *P. aeruginosa*. These data show that the structural similarities of *P. aeruginosa* and *E. coli* LPS do not stimulate cross-refractivity in this test system.

Our observation indicate that the refractivity of C3H/HeJ mice to bacterial endotoxins may be a phenomenon limited to LPS from enterobacteriaceae. We recognize that C3H/HeJ mice are in fact low responders to the biologic effects of enterobacterial endotoxins inasmuch as other investigators have found enterobacterial LPS to be toxic (1) and immunogenic (6) at high doses in these mice. The responses we found for the *P. aeruginosa* LPS are not entirely out of the range of responses reported by some for enterobacterial LPS responses in C3H/HeJ mice. However, in our hands the commercially purchased *E. coli* LPS produced numerous biologic responses in responder C3H/ANF mice and no responses in C3H/HeJ mice indicating that comparisons between different investigators utilizing slightly different systems need to be interpreted with caution. Nonetheless, partial refractivity may occur with LPS from other Gram-negative bacteria that are structurally similar but not identical to enterobacterial LPS, whereas no refractivity may be seen to a very different LPS, such as that from *B. abortus* (8, 9). We conclude that the refractivity to many of the endotoxin-induced biologic activities in C3H/HeJ mice is not a phenomenon generally applicable to LPS from all Gram-negative bacteria and that responses to structurally similar LPS may be seen in various assays of endotoxin responsiveness of C3H/HeJ mice.

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High-Molecular-Weight Polysaccharide Antigen from *Pseudomonas aeruginosa* Immunotype 2

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Previously, we isolated a high-molecular-weight immunogenic polysaccharide (designated PS) from *Pseudomonas aeruginosa* immunotype 1 (IT-1). The method which we used was modified to permit the isolation of a similar PS from *P. aeruginosa* IT-2. This antigen was composed primarily of carbohydrate, had a complex monosaccharide composition, including sugars not found in the lipopolysaccharide, and was nonpyrogenic in rabbits and nontoxic in mice at high doses. This material protected mice from challenges with live homologous cells. *P. aeruginosa* IT-2 PS gave a line of identity with the O side chain of the lipopolysaccharide, but differed from this polysaccharide in molecular weight, chemical composition, and ability to immunize mice actively. Lipopolysaccharide from *P. aeruginosa* IT-2 contained an immunological determinant not found on *P. aeruginosa* IT-2 PS, which was detected due to its stability during treatment with dilute alkali. Thus, we recovered a high-molecular-weight PS antigen from *P. aeruginosa* IT-2, which was serologically identical to the lipopolysaccharide O side chain but was chemically and physically distinct. Also, like *P. aeruginosa* IT-1 strains, *P. aeruginosa* IT-2 contains an alkali-stable immunodeterminant on the lipopolysaccharide that may represent a core-like antigen.

The high-molecular-weight polysaccharide designated PS which can be obtained from the outer cell surface or cultural supernatants of *Pseudomonas aeruginosa* immunotype 1 (IT-1) (7) actively protects mice from challenges with live homologous organisms (6). This antigen is composed principally of carbohydrate, has a molecular weight of approximately 1.5×10^5, is nonpyrogenic in rabbits at a dose of 25 μg/kg of body weight, and is nontoxic in mice at a dose of 400 mg/kg of body weight. The high molecular weight of PS, which is necessary for immunogenicity, and the low toxicity of this compound in animals suggest that it may be a candidate for a human vaccine.

PS appears to be a high-molecular-weight form of the polysaccharide side chain from *P. aeruginosa* IT-1 lipopolysaccharide (LPS) since PS and a polysaccharide which was obtained from LPS that had been hydrolyzed with acetic acid (O side chain) appeared to be immunologically identical (7). About 20% of the weight of *P. aeruginosa* IT-1 LPS can be accounted for by an O side chain fraction that elutes in the void volumes of Sephadex G-100 columns, and another 50% can be accounted for by an immunologically identical but smaller polysaccharide fraction. The LPS and PS from *P. aeruginosa* IT-1 differ chemically, since PS contains galactose and arabinose and LPS does not (7).

A systematic study of the PS antigens in different strains of *P. aeruginosa* led to the isolation and characterization of PS from *P. aeruginosa* IT-2. As described here, *P. aeruginosa* IT-2 PS is also immunogenic and nontoxic in mice, is nonpyrogenic in rabbits, and exhibits serological cross-reactions with *P. aeruginosa* IT-2 LPS.

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* IT-2 (strain 05142) and IT-6 (strain 01544) were obtained from M. Fisher, Parke, Davis & Co., Detroit, Mich.

Preparation of PS. *P. aeruginosa* IT-2 PS was prepared from 10-liter cultures of *P. aeruginosa* grown in Trypticase soy broth (Difco Laboratories, Detroit, Mich.) containing 3% glycerol. Each culture was grown in a Microferm fermentor (New Brunswick Scientific Co., New Brunswick, N.J.) for 72 h with stirring (200 rpm) and aeration (5 liters of air per h). Cetavalon (hexadecyltrimethylammonium bromide; final concentration, 0.5%; Fisher Scientific Co., Medford, Mass.) was added to the culture before centrifugation to remove the organisms. The supernatant was concentrated with a stacked ultrafiltration cell (Amicon

Corp., Lexington, Mass.) to a final volume of 800 ml by using PM-30 membranes. After precipitation of this concentrated supernatant with alcohol (80%, vol/vol), the crude material was collected by centrifugation and dissolved in phosphate-buffered saline, as described previously (7). This solution was then heated to 60 to 80°C, and Cetavalon (final concentration, 1.0%) was added. The precipitate was removed by centrifugation, and the supernatant was precipitated with alcohol (80%, vol/vol). The alcohol precipitate was collected by centrifugation and dissolved in phosphate-buffered saline; then it was subjected to a second series of heating, Cetavalon addition, centrifugation, alcohol precipitation of the supernatant, and centrifugation to collect the PS-containing material.

The addition of Cetavalon to hot preparations of crude concentrated culture supernatants allowed the elimination of the subsequent nuclease digestion steps described previously (7). Thus, after the two Cetavalon steps, the PS was purified by treating it with 1% acetic acid at 90°C for 18 h and extracting it with chloroform; this was followed by phenol extraction, and the compound was finally purified by Sephadex G-100 column chromatography, as described previously (7).

Preparation of LPS. LPS was isolated by the phenol-water extraction procedure of Westphal et al. (9). Low yields of LPS from the aqueous layer led us to investigate the phenol layer as a source of LPS. After the separation of the phenol and water layers, the phenol layer was dialyzed against running deionized water for 2 days, residual cells and denatured proteins were removed by centrifugation at 10,000 rpm for 30 min, and the LPS was recovered by ultracentrifugation at 33,000 rpm for 3 h. Further processing of the LPS recovered from the phenol phase was performed as described previously (7). We also attempted to extract the *P. aeruginosa* IT-2 LPS by the phenol-chloroform-petroleum ether method of Galanos et al. (3).

Preparation of O-specific polysaccharide. Isolated LPS was suspended in 1% acetic acid at a concentration of 10 mg/ml and then heated at 90°C for 6 h. The resulting lipid A precipitate was removed by centrifugation, and the supernatant was applied to a Sepharose CL-6B column (1.6 by 60 cm) by using phosphate-buffered saline as the eluant. The column eluate was monitored at 206 nm, and the serological activities of the optically active fractions were determined by the capillary precipitin reaction.

Alkali treatment of antigens and preparation of antisera. *P. aeruginosa* IT-2 PS and LPS were treated with 0.1 N NaOH at a concentration of 2 mg/ml at 56°C for 12 h. These solutions were neutralized with acid and buffer when they were used in immunological studies. Antisera to *P. aeruginosa* IT-2 cells, LPS, and PS were prepared as described previously (7).

Antisera to IT-2 alkali-treated LPS (A-LPS) were raised in New Zealand white rabbits that weighed 3 to 4 kg by an initial intramuscular injection of 0.5 mg of A-LPS in complete Freund adjuvant, followed 1 week later by a series of four intravenous injections of 0.5 mg of A-LPS in saline every 4 days. Blood was collected by cardiac puncture 6 days after the last injection.

Serological methods. Ouchterlony double diffusion and immunoelectrophoresis were performed as previously described (7). Hemagglutination of sheep erythrocytes (SRBC) coated with either LPS or A-LPS was performed in microtiter plates by using 25-μl volumes of serum dilutions and cells. SRBC were coated by suspending 0.12 ml of packed SRBC in 1.0 ml of saline containing 0.5 mg of antigen, followed by dropwise addition of 1.0 ml of a 0.01% chromium chloride solution. Titers were read as the reciprocals of the highest serum dilutions that caused agglutination of the SRBC. Each hemagglutination inhibition test was performed by incubating 25 μl of a serum dilution four times more concentrated than the titer with 25 μl of a solution containing the inhibitor to be tested; then the preparation was incubated at 37°C for 30 min, 25 μl of coated cells was added, and the preparation was incubated further at 37°C.

Chemical analysis. The chemical constituents and the monosaccharide and lipid compositions of the PS, LPS, and O side chain polysaccharide antigens were determined as described previously (7).

Molecular weight determinations. The molecular weights of the PS and O side chain polysaccharides were determined with a Waters Associates high-performance liquid chromatography system by using two protein I-250 sizing columns in series and 0.1 M phosphate buffer as the eluant. Dextran polymers of known molecular weights (Pharmacia Co., Uppsala, Sweden) were used as standards. The molecular weights of unknowns were determined by calculating linear regression plots of the elution volumes versus $\log_{10}$ molecular weights of the standards. The coefficient correlation for this formula was 0.95 over the molecular weight range from 10,000 to 250,000.

Animal studies. ICR mice obtained from the Walter Reed Army Institute of Research animal colony and CD-1 mice obtained from Charles River Laboratories, Wilmington, Mass., were used for active immunization and passive transfer experiments, as previously described (6). The pyrogenicity in rabbits and toxicity in mice of *P. aeruginosa* IT-2 PS were also determined as described previously (7).

Adsorption of antisera for passive transfer. Rabbit antisera to *P. aeruginosa* IT-2 PS, LPS, and A-LPS were adsorbed with these antigens by incubating 1 ml of antiserum with 1 mg of the absorbing antigen at 37°C for 2 h; this was followed by a 48-h incubation at 4°C and the hemagglutination removal of any precipitate by centrifugation. The sera were then tested with the hemagglutination assay and readsorbed as described above if any hemagglutination activity was still present.

RESULTS

Isolation of *P. aeruginosa* IT-2 PS, LPS, and O-side chain polysaccharide. The use of the procedures described above for isolating PS antigen from supernatants of *P. aeruginosa* IT-2 cultures consistently yielded a product which had a high carbohydrate content, eluted in the void volume of a Sephadex G-100 column, and gave a single precipitin line in Ouchterlony dou-

ble-diffusion gels. Attempts to isolate PS antigen from agar cultures of *P. aeruginosa* IT-2 were generally unsuccessful, indicating that production of the high-molecular-weight PS was facilitated by growth in liquid media. The yields of *P. aeruginosa* IT-2 PS were lower than the yields of *P. aeruginosa* IT-1 PS; the average yield was 5 mg of PS per liter of medium. To eliminate detectable LPS from PS preparations, it was necessary to hydrolyze the LPS into its lipid and polysaccharide components with acetic acid. As found previously with *P. aeruginosa* IT-1 PS (7), *P. aeruginosa* IT-2 LPS could not be eliminated from the PS preparation by ultracentrifugation, column chromatography in the presence or absence of disaggregating buffers, or ion-exchange chromatography. *P. aeruginosa* IT-2 PS was serologically active after this treatment and remained stable to acetic acid hydrolysis at 95°C for up to 72 h.

Attempts to isolate LPS from *P. aeruginosa* IT-2 by phenol-water extraction (9) of cells generally gave poor yields of antigen in the aqueous phase. The LPS of this strain was best recovered from the phenol phase. The presence of *P. aeruginosa* IT-2 LPS in the phenol phase is unique to the LPS of this *P. aeruginosa* immunotype (unpublished data). The use of the phenol-chloroform-petroleum ether method of Galanos et al. (3) for extracting LPS from *P. aeruginosa* IT-2 was unsuccessful.

O-side chain polysaccharide was obtained from acetic acid-hydrolyzed LPS as a single serologically active peak which eluted from a Sepharose CL-6B column (1.6 by 60 cm) with a K_{av} of 0.52. Two other peaks were also detected in column eluates of acetic acid-hydrolyzed LPS. One of these was a very small peak which eluted in the void volume and was presumably nonhydrolyzed LPS; this peak was not characterized further. The second peak was a peak of carbohydrate-containing material that eluted in the bed volume and was neither serologically active nor precipitable with alcohol but was dialyzable; this peak had a molecular weight of less than 10,000, as determined by high-performance liquid chromatography.

Characterization of *P. aeruginosa* IT-2 PS and LPS. Table 1 shows the chemical compositions of the PS and LPS isolated from *P. aeruginosa* IT-2. The PS was composed almost exclusively of carbohydrate but did contain low levels of nucleic acids and protein. The water content of PS was 18.4%, as determined by a Karl Fischer titration (7). No lipid was detected in *P. aeruginosa* IT-2 PS by quantitative gas-liquid chromatography (limit of detection, 1 part in 1,000). On the other hand, LPS contained lipid, protein, nucleic acid, phosphate, and carbohydrate. The lipid and carbohydrate contents of our *P. aeruginosa* IT-2 LPS preparation were consistent with the contents reported by other investigators (4, 10).

TABLE 1. *Compositions of P. aeruginosa IT-2 PS and LPS*

Component	% By weight in:	
	PS	LPS
Carbohydrate	83.4	72
Lipid	0	12.2
Nucleic acid	1.2	2.2
Protein	0.8	5.8
Phosphate[a]	0	2.4
Water	18.4	

[a] The material for phosphate determinations was freed of nucleic acid by passage over an ion-exchange column.

Monosaccharide compositions of *P. aeruginosa* IT-2 PS, LPS, and O-side chain polysaccharide. Gas-liquid chromatographic and colorimetric analyses of the monosaccharide compositions of *P. aeruginosa* IT-2 PS, intact *P. aeruginosa* IT-2 LPS, and the O-side chain polysaccharide portion of the LPS showed that the LPS contained rhamnose, mannose, glucose, 2-acetamido-2,6-dideoxygalactose, galactosamine, glucosamine, heptose, and 2-keto-3-deoxyoctulosonic acid, whereas PS contained mannose, glucose, rhamnose, glucosamine, xylose, galactose, and arabinose; the latter three sugars were not detected in LPS (Table 2). The O-side chain polysaccharide portion of the LPS contained only rhamnose, mannose, and glucose.

Molecular weights of *P. aeruginosa* IT-2 PS and O-side chain polysaccharide from *P. aeruginosa* IT-2 LPS. High-performance liquid chromatography of *P. aeruginosa* IT-2 PS on a series of two I-250 protein-sizing columns showed that this preparation eluted as a single homogeneous peak with a molecular weight of 2.25×10^5. The serologically active O-side chain polysaccharide had a molecular weight of 0.3×10^5.

Chromatography of *P. aeruginosa* IT-2 PS in the presence of 3% sodium deoxycholate on a Sephadex G-100 column (2.6 by 100 cm) did not change the elution pattern of the PS.

Animal toxicity of *P. aeruginosa* IT-2 PS. Injection of up to 25 μg of *P. aeruginosa* IT-2 PS per kg into rabbits elicited no pyrogenic response(<0.1°C increase in 3 h). Similarly, 400 mg of *P. aeruginosa* IT-2 PS per kg injected intraperitoneally into mice elicited no signs of toxicity or symptoms of endotoxin poisoning, and the mice gained weight normally.

Serological relationship of *P. aeruginosa* IT-2 PS and LPS. Immunoelectrophoresis of *P.*

TABLE 2. *Molar ratios of sugars in P. aeruginosa IT-2 PS and LPS*

Prepn	Molar ratio of:[a]										
	Arabinose	Rhamnose	Xylose	Mannose	Galactose	Glucose	KDO	Gluam	Galam	Hep	Dideoxygal
PS	0.74	0.19	0.10	0.34	2.23	1.00	0	0.13	0	0	P[b]
LPS	0	0.34	0	0.15	0	1.00	0.05	0.05	0.05	0.04	P
O-side chain polysaccharide from LPS	0	0.36	0	0.34	0	1.00	0	0	0	0	0

[a] Molar ratio per mole of glucose. Abbreviations: KDO, 2-keto-3-deoxyoctonoate; Gluam, 2-acetamido-2-deoxyglucose; Galam, 2-acetamido-2-deoxygalactose; Hep, L-glycero-D-mannoheptose; Dideoxygal, 2-acetamido-2,6-dideoxygalactose.

[b] P, Present. Lack of a standard prohibited assignment of a molar value.

aeruginosa IT-2 PS showed that this antigen migrated toward the cathode when it was electrophoresed for 45 min (Fig. 1). This is the opposite polarity of migration shown by *P. aeruginosa* IT-1 PS. *P. aeruginosa* IT-2 LPS did not migrate after 2 h of electrophoresis.

In immunodiffusion gels, *P. aeruginosa* IT-2 PS gave a single precipitin line when it was tested against antiserum to intact *P. aeruginosa* IT-2 cells (Fig. 2). Acetic acid hydrolysis of *P. aeruginosa* IT-2 LPS released into the solution an antigen that gave a reaction of identity with *P. aeruginosa* IT-2 PS in immunodiffusion gels. A similar line of identity was generated with *P. aeruginosa* IT-2 LPS solutions that stood in phosphate-buffered saline at room temperature for 1 to 2 weeks (Fig. 2).

Table 3 shows the hemagglutination assay titers of antisera prepared against *P. aeruginosa* IT-2 cells, *P. aeruginosa* IT-2 LPS, *P. aeruginosa* IT-2 A-LPS, and *P. aeruginosa* IT-2 PS when these preparations were tested versus SRBC coated with either LPS or A-LPS. Attempts to coat SRBC with *P. aeruginosa* IT-2 PS by using either chromium chloride linking or steroyl chloride derivitization of PS (7) were unsuccessful. However, since acid-hydrolyzed LPS and PS gave a line of identity in immunodiffusion gels, it was possible to use LPS-sensitized SRBC to measure antibodies to PS. The specificity of this reaction was confirmed by using PS as an inhibitor in a hemagglutination inhibition assay.

We found that alkali treatment (0.1 N NaOH, 37°C, 2 h) destroyed the serological activity of *P. aeruginosa* IT-1 PS and that the determinants shared with *P. aeruginosa* IT-1 LPS were also destroyed by this treatment. We did not observe this phenomenon with *P. aeruginosa* IT-2 LPS, even after alkali treatment at 56°C for 12 h. Antiserum to *P. aeruginosa* IT-2 PS reacted at the same titer with A-LPS-coated

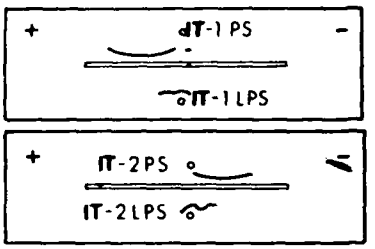

FIG. 1. *Immunoelectrophoretic patterns of PS and LPS from P. aeruginosa IT-1 and IT-2.*

SRBC and with intact LPS-coated SRBC (Table 3). However, alkali treatment of *P. aeruginosa* IT-2 PS at 56°C for 12 h did alter the inhibitory activity of this PS in serological assays specific for the PS determinants, indicating that when the PS determinants were freed from the LPS, they became alkali labile (Table 4). Antiserum to *P. aeruginosa* IT-2 A-LPS (56°C, 12 h) reacted with SRBC coated with A-LPS (Table 3). This reactivity could be inhibited by both intact LPS and A-LPS but not by PS, acetic acid-hydrolyzed LPS, or alkali-treated PS (Table 4). These results indicated that although *P. aeruginosa* IT-2 A-LPS and *P. aeruginosa* IT-2 PS had serologically active determinants in common, the immunogenicity of these determinants was destroyed by alkali treatment. This allowed us to detect alkali-stable determinants on the LPS by using an assay in which antisera to A-LPS and A-LPS-coated SRBC were used.

Active immunization of mice with *P. aeruginosa* IT-2 PS, LPS, O-side chain polysaccharide, alkali-treated PS, and A-LPS. *P. aeruginosa* IT-2 PS induced protective immunity in mice that were given 10 to 50 μg of *P. aeruginosa* IT-2 PS intraperitoneally and challenged 7 days later with 2.6 100% lethal doses of live *P. aeruginosa* IT-2 (Table 5). These immunizations were not effective in protecting

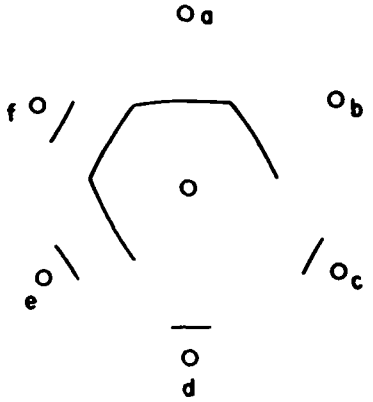

FIG. 2. *Immunodiffusion gel patterns obtained with P. aeruginosa IT-2 PS, acetic acid-hydrolyzed LPS, and LPS rehydrated and left to stand at room temperature versus antiserum to P. aeruginosa IT-2 (center well). The antigens in the outer wells were as follows: well a, PS (1 mg/ml); well b, acetic acid-hydrolyzed LPS (1 mg/ml); well c, freshly rehydrated LPS (1 mg/ml); well d, LPS rehydrated in solution for 3 days (1 mg/ml); well e, LPS in solution for 7 days (1 mg/ml); well f, LPS in solution for 14 days (1 mg/ml).*

TABLE 3. *Hemagglutination titers of antisera to P. aeruginosa IT-2 cells, PS, LPS, and A-LPS when tested versus SRBC coated with either LPS or A-LPS*

Antiserum to	Titer when tested against SRBC coated with:	
	LPS	A-LPS
Cells	1,024[a]	2,048
PS	64	64
LPS	1,024	2,048
A-LPS	64	1,024

[a] Reciprocal of the twofold serum dilution that produced positive agglutination of antigen-coated SRBC.

mice against challenge with *P. aeruginosa* IT-6. LPS from *P. aeruginosa* IT-2 was effective in protecting mice at a level of 0.10 μg/mouse against a similar challenge (Table 5); again, no protection against challenge with *P. aeruginosa* IT-6 was observed. Isolated O-side chain polysaccharide was not effective in inducing protection at doses of up to 500 μg/mouse. Alkali treatment of *P. aeruginosa* IT-2 PS (56°C, 12 h) destroyed the protective efficacy of this compound at doses of up to 500 μg/mouse, whereas alkali treatment of *P. aeruginosa* IT-2 LPS raised the minimum effective protective dose of this compound to 1.0 μg/mouse (Table 5).

Table 6 shows the enhancement of nonspecific resistance to challenges with live cells 24 h after immunization with *P. aeruginosa* IT-2 PS and LPS. Doses of PS of up to 500 μg/mouse provided no protection against challenge with live cells, whereas doses of 1 and 10 μg of LPS per mouse provided a minimal level of nonspecific resistance. Higher doses of LPS caused severe endotoxin poisoning symptoms in the animals.

Passive transfer studies. Confirmation of the presence of cross-reactive determinants on *P. aeruginosa* IT-2 PS and LPS was obtained by passively transferring rabbit antisera raised

TABLE 4. *Inhibitory activities of PS, alkali-treated PS, LPS, A-LPS, and LPS O side chain in serological assays*

Inhibitor	Serum inhibited	Activity of antigen on SRBC	
		LPS	A-LPS
PS	Anti-LPS	9[a]	7
Alkali-treated PS	Anti-LPS	—[b]	—
LPS	Anti-A-LPS	10	8
A-LPS	Anti-A-LPS	7	9
PS	Anti-A-LPS	—	—
O side chain	Anti-A-LPS	—	—
Alkali-treated PS	Anti-A-LPS	—	—

[a] Reciprocal of the $\log_2$ dilution of a 1-mg/ml solution that produced inhibition of 4 hemagglutination units of serum.

[b] —, Inhibitory activity at 1 mg/ml.

TABLE 5. *Active immunization of mice with P. aeruginosa IT-2 PS, alkali-treated PS, LPS, O side chains, and A-LPS*

Immunogen	Amt (μg)	Challenge strain	No. of LD_{100}[a]	% Protected[b]	*P* value[c]
PS	0	IT-2	2.6	0	
	10	IT-2	2.6	50	0.016
	25	IT-2	2.6	70	0.002
	50	IT-2	2.6	80	<0.001
	50	IT-6	1.8	0	
LPS	0	IT-2	2.9	0	
	0.01	IT-2	2.9	30	0.105
	0.10	IT-2	2.9	100	<0.001
	0.10	IT-6	1.6	0	
O side chains	0	IT-2	2.6	0	
	10	IT-2	2.6	0	
	100	IT-2	2.6	0	
	500	IT-2	2.6	0	
A-LPS	0	IT-2	2.9	0	
	0.10	IT-2	2.9	10	0.500
	1.0	IT-2	2.9	70	0.002
	10.0	IT-2	2.9	60	0.005
Alkali-treated PS	500	IT-2	2.9	0	

[a] LD_{100}, 100% lethal dose. The 100% lethal doses for *P. aeruginosa* IT-2 and IT-6 were 10^7 and 10^8 cells per mouse, respectively.

[b] Ten mice were used in each experiment.

[c] *P* values were calculated by the Fisher exact test.

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Lipopolysaccharide and High-Molecular-Weight Polysaccharide Serotypes of *Pseudomonas aeruginosa*

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The serotype distribution of bacteremic and nonbacteremic clinical isolates of *Pseudomonas aeruginosa* in relation to the Fisher immunotyping scheme, the International Antigenic Typing System (IATS), and high-molecular-weight polysaccharide determinants was investigated. Of 281 bacteremic isolates, 273 (97.2%) were serotyped by one of the seven IATS specificities that correspond to a Fisher lipopolysaccharide/high-molecular-weight polysaccharide specificity. In contrast, these seven serotypes accounted for only 68.5% of 124 nonbacteremic clinical isolates. Review of the reported serotype distribution of *P. aeruginosa* isolates in Europe further supported the finding of a limited serotype distribution among bacteremic clinical isolates. Fifteen of the 17 IATS serotypes were found among all of the strains of *P. aeruginosa* serotyped, an indication that most of the IATS serotypes are present in the United States. Thus, only certain lipopolysaccharide immunotypes of *P. aeruginosa* occur as clinical bacteremic isolates, and a multivalent, high-molecular-weight polysaccharide vaccine directed at the lipopolysaccharide type determinants of *P. aeruginosa* has potential usefulness.

Serotyping schemes for *Pseudomonas aeruginosa* have been proposed by many investigators from all over the world [1–5]. Because of the ubiquity of *P. aeruginosa* in the environment, a means of differentiating strains has been thought necessary for understanding the environmental and epidemiologic distribution of this organism. Most of the serotyping schemes have been based on heat-stable antigens. For two of these systems, Habs [6] and Fisher immunotype [7], the antigenic determinant relative to the serotype schemes has been shown to reside on the lipopolysaccharide (LPS). The Habs system has now been proposed as the basis for an international system termed the International Antigenic Typing System (IATS); it incorporates the 12 serotypes of the Habs system and five additional types [8].

Although numerous investigators have presented data regarding the serotype distribution of *P. aeruginosa* from clinical [9, 10] and environmental [11] sources, most have concluded that serotyping of *P. aeruginosa* offered little means to understanding the epidemiology of the organism [8]. Most of these studies showed an association of a few types with various clinical states or environmental niches [9, 11], but no conclusive data regarding either serotype specificity of organisms for clinical conditions or spread of certain serotypes in a hospital environment were seen. It is curious that the Fisher system, consisting of seven immunotypes, has been reported in the United States to type 92%–98% of *P. aeruginosa* isolates [9, 10], whereas in other countries the 17 serotypes of the IATS system are needed for comprehensive typing of most isolates. The seven Fisher type determinants are represented among the 17 IATS types [12], an indication that they share similar specificities.

We have been investigating a high-molecular-weight polysaccharide (PS) antigen, purified from cultural supernatants of *P. aeruginosa*, as a possible vaccine candidate. We have shown [13, 14] that the high-molecular-weight PSs purified from *P. aeruginosa* Fisher types 1 and 2 share serologic determinants with the O side-chain antigen of their respective LPSs. The high-molecular-weight PS antigens differ from the LPS antigens in terms of chemical composition, monosaccharide constit-

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uents, molecular size, and toxicity in animals [13, 14]. Furthermore, these high-molecular-weight PS antigens are immunogenic in animals and actively protect mice from challenge with live organisms [14, 15]. The high-molecular-weight PS antigens most likely represent a form of the LPS type determinant that lacks toxicity but retains immunogenicity. Recently the type 1 high-molecular-weight PS has been shown to be immunogenic in humans, inducing both binding and opsonic antibody [16]. Unlike LPS vaccines [17] from *P. aeruginosa*, the high-molecular-weight PS vaccine was almost devoid of toxicity [16].

Therefore, it was of interest to determine the serologic diversity of clinical isolates of *P. aeruginosa* in relation to the serologic specificities of high-molecular-weight PSs and to correlate these specificities with those of the Fisher LPS system [1] and the IATS [8]. We were interested in ascertaining the diversity of serotype antigens among bacteremic and nonbacteremic strains of *P. aeruginosa* with the goal of investigating how many components a high-molecular-weight PS vaccine may ultimately need to contain so that the serotype specificity of bacteremic isolates of *P. aeruginosa* will be comprehensively covered.

Materials and Methods

Bacterial strains. Strains of *P. aeruginosa* for production of antisera were the prototype strains of the Fisher LPS typing system, provided by Dr. M. W. Fisher, Parke-Davis Co., Detroit. Strains of the IATS system were supplied by Dr. T. Pitt of the Public Health Laboratory, Colindale, London, England. Clinical isolates were obtained from the following sources: University of California at Los Angeles Hospital, courtesy of Dr. Lowell Young; Brigham and Women's Hospital, Boston, courtesy of Dr. Tom O'Brien and Elaine Gileece; Walter Reed Army Hospital, Washington, D.C., courtesy of Dr. Alan Cross; New England Deaconess Hospital, Boston, courtesy of Dr. Paola Digirolami; Massachusetts General Hospital, Boston, courtesy of Dr. George Jacoby; West Roxbury Veterans Administration Hospital, Boston, courtesy of Dr. Dennis Evans; and the Beth Israel Hospital, Boston, courtesy of Dr. Ira Tager. All isolates were confirmed as being *P. aeruginosa* in the original laboratory and were checked by us for colonial morphology on agar, Gram-stain reaction, growth at 42 C, and growth on cetrimide agar.

Antigens. The antigens used for immunization of rabbits were the LPSs of the seven Fisher immunotypes extracted with phenol and water [13, 14] and the high-molecular-weight PS antigens from these strains [13, 14].

Antisera. Antisera to the LPS and high-molecular-weight PS antigens from the seven Fisher immunotype strains were produced in New Zealand white rabbits weighing 3–4 kg each. High-molecular-weight PS or LPS (0.5 mg/ml) was mixed with an equal volume (0.5 ml each) of complete Freund's adjuvant and injected into the footpad of a rabbit. The antigen (1.0 mg/ml) in 0.9% NaCl was also inoculated iv in 0.5-ml amounts every day for one week. After the animals were rested for one week, the above immunization schedule was repeated. Five days after the final injection, the rabbits were bled by cardiac puncture, and the serum was prepared from clotted blood.

Table 1. Agglutination titers of three sets of antisera to the 17 International Antigenic Typing System (IATS) strains of *Pseudomonas aeruginosa*.

	IATS serotype no.																
Rabbit antiserum to	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
IATS strains	8	20	30	4	30	20	40	4	40	30	40	20	40	40	4	4	30
Fisher lipopolysaccharide	8	2*	...	...	32	4	...	8	...	4	4	...	...	...	...	...	...
Fisher high-molecular-weight polysaccharide	2	8	...	...	16	4	...	4	...	2	4†	...	...	...	...	...	...

NOTE. Data are the reciprocal of the serum dilution that agglutinated with the strain from which the immunizing antigen was derived.

* This serum was adsorbed with Fisher type 7 organisms.

† This serum was adsorbed with Fisher type 2 and type 7 organisms.

Agglutinations were performed using antisera made monospecific to the strain from which the immunizing LPS or high-molecular-weight PS was isolated by dilution of the serum to the agglutination titer against the immunizing strain and adsorption with cross-reactive organisms of the other six Fisher immunotypes until the cross-reactivity was eliminated. Most of the antisera were monospecific at the agglutination titer, except for the antisera to the Fisher type 3 LPS and type 2 PS. Antisera to the IATS strains were purchased from Difco Laboratories, Detroit.

Agglutination assay. For serotyping of strains, the growth from an overnight culture of *P. aeruginosa* at 37 C on trypticase soy agar plates was suspended in 0.9% NaCl to an OD at 650 nm of 2.0. This suspension (15 μl) was mixed with 15 μl of the typing serum in a microtiter plate (Microbiological Associates, Bethesda, Md.), and agglutination was observed within 15 min. Controls included 0.9% NaCl with suspended organisms and normal rabbit serum with suspended organisms. Strains that did not give a smooth suspension in 0.9% NaCl but instead looked granular were repeatedly passed through a 1-ml syringe to which a 27-gauge needle was attached to achieve a smooth suspension.

Results

Antisera standardization. Agglutination titers of antisera to the 17 IATS strains that were prepared to whole organisms (Difco antisera), Fisher immunotype LPS, or Fisher immunotype high-molecular-weight PS are shown in table 1. Most of the sera were monospecific at the agglutination titer, except for antisera to Fisher type 2 high-molecular-weight PS and type 3 LPS. Both of these sera required adsorption with Fisher type 7 organisms, and antiserum to type 3 LPS needed to be adsorbed with type 2 organisms. All of the Difco antisera to the IATS strains were monospecific at the agglutination titer. However, some of these sera had a titer less than the manufacturer's recommended use of a 1:10 dilution.

Correlation of the serotype specificities of the Difco antisera to IATS strains, antisera to Fisher immunotype LPS, and antisera to Fisher immunotype high-molecular-weight PS was undertaken by serotyping 137 isolates of *P. aeruginosa* with each set of antisera. Previous reports [8, 12] correlating the Fisher typing scheme with the IATS looked only at the agglutination patterns of the prototype strains from these classifications; correlations were not extended to other strains. Of strains typable by one of the seven IATS serotypes that corresponded to a Fisher LPS immunotype, 90% were agglutinated by the corresponding antiserum to Fisher immunotype LPS (table 2). None of the IATS serotypes that do not correspond to a Fisher LPS immunotype was agglutinated by the antisera to Fisher immunotype LPS. Correlation of the specificities of the Fisher LPS immunotype and high-molecular-weight PS serotype showed that 97 of 100 strains serotyped by antiserum to purified LPS were also serotyped by the corresponding antiserum to high-molecular-weight PS. Thus, the high-molecular-weight PS showed the same type specificity as LPS.

Serotyping of clinical isolates. We obtained 281 distinct bacteremic isolates of *P. aeruginosa* from four hospital laboratories for the interval 1973–1980 (table 3). No effort was made to ascertain the underlying host condition associated with the bacteremia due to *P. aeruginosa*. The serotype distribution of these strains (table 4) revealed that

Table 2. Correlation of the International Antigenic Typing System (IATS) serotype with the Fisher lipopolysaccharide (LPS) and high-molecular-weight polysaccharide (PS) immunotype among 137 isolates of *Pseudomonas aeruginosa*.

Serotype by IATS (no. of strains)	Immunotype by Fisher LPS/PS system	No. (%) with serotype	
		Same	Different
1 (15)	4	15 (100)	0
2 (15)	3	12 (80)	3 (20)*
3 (5)	...	0	...
4 (5)	...	0	...
5 (10)	7	8 (80)	2 (20)
6 (15)	1	13 (87)	2 (13)
7 (1)	...	0	...
8 (15)	6	12 (80)*	3 (20)
9 (5)	...	0	...
10 (15)	5	15 (100)	...
11 (15)	2	15 (100)*	...
12 (5)	...	0	...
13 (4)	...	0	...
14 (2)	...	0	...
15 (4)	...	0	...
16 (5)	...	0	...
17 (1)	...	0	...

* These groups each contained one isolate for which the Fisher LPS type did not correspond to the Fisher high-molecular-weight PS type.

273 (97.2%) of these isolates were typed by antiserum to one of the IATS serotypes that correspond to a Fisher LPS immunotype. Three of the remaining eight isolates were not typable, and the other five were distributed among IATS serotypes 3, 4, 9, and 16. IATS combination serotype 2,5 was treated as a separate category, which corresponds to Fisher immunotype 3,7.

The serotype distribution of 124 nonbacteremic isolates of *P. aeruginosa* from a variety of clinical sources is also shown in table 4. In contrast to bacteremic isolates, only 68.5% of nonbacteremic strains of *P. aeruginosa* occurred among the seven IATS serotypes corresponding to a Fisher LPS immunotype. None of the nonbacteremic isolates was IATS serotype 5 (Fisher type 7). The remaining 31.5% of nonbacteremic isolates were randomly distributed among the IATS serotypes that do not correspond to a Fisher immunotype, except that no IATS serotype 7 or 17 was found.

Next we looked at the serotype distribution of bacteremic and nonbacteremic isolates of *P. aeruginosa* from the same hospital over the same interval. These isolates all came from different patients, and none of the bacteremic isolates came from a patient with a nonbacteremic isolate. Of 26 bacteremic isolates, 24 (92.3%) were typed by antiserum to one of the IATS serotypes that correspond to a Fisher immunotype (table 5). In contrast, 24 (75%) of 32 nonbacteremic isolates were distributed among these same seven serotypes, whereas the other 25% were distributed among five IATS serotypes that do not correspond to a Fisher immunotype.

Discussion

Although serotyping of isolates of *P. aeruginosa* is thought to be of limited value in deriving epidemiologic information about the organism [8], the present data clearly indicate the limited serotype distribution among bacteremic isolates of *P. aeruginosa*. The correlation of the specificities of the Fisher LPS immunotype and high-molecular-weight PS serotype with the specificity of the IATS serotype among clinical isolates of *P. aeruginosa* indicates that the high-molecular-weight PS antigen represents an immunogenic, nontoxic form of the LPS type determinant. The fact that the high-molecular-weight PS antigens are a nontoxic form of the LPS type determinants is further

Table 3. Source of clinical isolates of bacteremic strains of *Pseudomonas aeruginosa*.

Source	No. of strains	Years
Brigham and Women's Hospital, Boston	152	1973–1980
Walter Reed Army Hospital, Washington, D.C.	11	1975–1978
Massachusetts General Hospital, Boston	7	1980
University of California at Los Angeles Hospital	111	1973–1979

encouragement as to the vaccine potential of high-molecular-weight PS because antibody to these determinants is the important correlate of human immunity to infection with *P. aeruginosa* [10, 18]. Pollack and Young [19] showed that 85% of pa-

Table 4. Serotype distribution of 281 bacteremic and 124 nonbacteremic isolates of *Pseudomonas aeruginosa*.

IATS serotype no.	Fisher LPS immunotype no.	Isolates	
		Bacteremic*	Non-bacteremic†
1	4	41 (14.6)	7 (5.6)
2	3	32 (11.4)	5 (4.0)
3	. . .	2 (<0.1)	3 (2.4)
4	. . .	1 (<0.1)	5 (4.0)
5	7	10 (3.6)	0
6	1	79 (28.1)	26 (21.0)
7	. . .	0	0
8	6	18 (6.4)	5 (4.0)
9	. . .	1 (<0.1)	4 (3.2)
10	5	45 (16.0)	11 (8.8)
11	2	37 (13.2)	26 (21.0)
12	. . .	0	5 (4.0)
13	. . .	0	4 (3.2)
14	. . .	0	2 (0.8)
15	. . .	0	4 (3.2)
16	. . .	1 (<0.1)	5 (4.0)
17	. . .	0	0
2,5	3,7	11 (3.9)	5 (4.0)
MT‡	. . .	0	4 (3.2)
NT§	. . .	3 (0.1)	3 (2.4)

NOTE. Data are no. serotyped (%). IATS = International Antigenic Typing System; LPS = lipopolysaccharide.

* Of the 281 isolates serotyped, 273 (97.2%) were one of the seven IATS serotypes (including serotype 2,5) corresponding to a Fisher LPS immunotype.

† Of the 124 isolates serotyped, 85 (68.5%) were one of the seven IATS serotypes corresponding to a Fisher LPS immunotype.

‡ MT = multiple serotypes.

§ NT = nontypable.

Table 5. Serotypes of bacteremic and nonbacteremic isolates of *Pseudomonas aeruginosa* isolated from different patients in the same hospital over the same interval.

IATS serotype no.	Fisher LPS immunotype no.	No. (%) of strains serotyped* Bacteremic	Nonbacteremic
1	4	1 (3.8)	1 (3.1)
2	3	3 (11.5)	0
3	. . .	2 (7.7)	0
4	. . .	0	1 (3.1)
5	7	2 (7.7)	0
6	1	2 (7.7)	8 (25.0)
7	. . .	0	0
8	6	2 (7.7)	2 (6.2)
9	. . .	0	1 (3.1)
10	5	5 (19.2)	3 (9.4)
11	2	7 (26.9)	10 (31.3)
12	. . .	0	1 (3.1)
13	. . .	0	0
14	. . .	0	1 (3.1)
15	. . .	0	1 (3.1)
16	. . .	0	3 (9.4)
17	. . .	0	0
2,5	3,7	2 (7.7)	0
MT†	. . .	0	0
NT‡	. . .	0	0

* Twenty-four (92.3%) of 26 bacteremic isolates were of an International Antigenic Typing System (IATS) serotype that corresponds to one of the seven Fisher lipopolysaccharide (LPS) immunotypes, whereas 24 (75%) of 32 nonbacteremic isolates were of an IATS serotype that corresponds to one of the seven Fisher LPS immunotypes.

† MT = multiple serotypes.

‡ NT = nontypable.

tients with high titers of antibody to LPS in acute-phase serum survived infection with *P. aeruginosa* vs. 48% of persons with low titers of antibody to LPS. The encouraging results in active immunization studies of humans using a heptavalent LPS vaccine to the seven Fisher LPS immunotypes have indicated the efficacy of antibody to this serologic determinant in protecting the patient at risk from *P. aeruginosa*-associated death [20–24].

The development of LPS type-specific antibodies after infection with *P. aeruginosa* has also provided evidence for the role of serotype-specific immunity. Crowder et al. [18] showed that after infection with *P. aeruginosa* the sera of 15 of 22 patients contained at least one precipitin for LPS antigens, whereas sera from normal subjects lacked precipitins for *P. aeruginosa* LPS. Young et al. [25] showed that 91% of patients recovering from *P. aeruginosa* bacteremia or other deep infections had antibody to LPS antigens. None of 50 normal control subjects and none of 47 patients recovering from bacteremia caused by other gram-negative bacteria possessed these precipitins for LPS. These data clearly indicate an immune response of infected individuals to LPS type determinants after infection with *P. aeruginosa*.

Our present data indicate that among bacteremic isolates of *P. aeruginosa* in the United States, an overwhelming majority (97.2%) are one of the seven IATS serotypes that correspond to a Fisher LPS immunotype. Furthermore, the high-molecular-weight PS antigens, which cross-react serologically with LPS determinants [13, 14], induced an antibody response in rabbits identical to that seen with LPS immunization. The resultant agglutination pattern of organisms with antisera to high-molecular-weight PS is essentially identical with that to antisera to LPS. Thus, because high-molecular-weight PS antigens are capable of inducing type-specific antibodies to *P. aeruginosa* and because there are a limited number of important serotypes in bacteremic isolates of *P. aeruginosa*, the development of a multivalent, high-molecular-weight PS vaccine appears feasible.

On the basis of these studies, the valency of this vaccine would appear to be seven. In Denmark [26] and the Federal Republic of Germany [27], the seven IATS serotypes that correspond to a Fisher LPS immunotype accounted for 90.9% and 88%, respectively, of blood and cerebrospinal fluid isolates of *P. aeruginosa*; when two other IATS serotypes, 3 and 4 (which do not correspond to a Fisher serotype), are included, then 96.9% and 100%, respectively, of blood and cerebrospinal fluid isolates are accounted for. This number may represent an upper limit to the valency of a vaccine directed at serotype determinants of *P. aeruginosa*.

The occurrence of IATS serotypes of *P. aeruginosa* other than the seven that correspond to the Fisher LPS immunotypes among nonbacteremic isolates in our study indicates that the serotype distribution of *P. aeruginosa* in the United States is not limited. Furthermore, in the studies of Mikkelsen [26], Ullmann and Schmulling [27], and Southern et al. [28], IATS serotypes 7 and 12–15 were never isolated from blood. Data regarding serotypes 16 and 17 were not presented in these

studies. We found that 14 of 17 IATS serotypes occur among nonbacteremic isolates of *P. aeruginosa* in the United States and that one of the three serotypes not found among nonbacteremic isolates (IATS serotype 5) occurs among bacteremic isolates. In a single hospital monitored over a single interval, five IATS serotypes that do not correspond to a Fisher LPS immunotype occurred among nonbacteremic isolates, but these serotypes did not occur among bacteremic isolates. Thus, *P. aeruginosa* IATS serotypes 1-6, 8, 10, and 11 appear to represent markers for virulent serotypes capable of causing bacteremic disease. This situation is analogous to disease caused by *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae*, in which only certain serotypes are associated with pathogenicity in humans. However, for these examples and for *P. aeruginosa* these surface serotype markers are not considered pathogenic. Indeed, for some of these organisms, the surface serotype determinants (in the form of purified capsular polysaccharides) are used as human vaccines. Because both organism factors and host resistance determine whether a disease will occur, the serotype determinants associated with bacteremic strains of *P. aeruginosa* only indicate those strains with pathogenic potential, which we defined as causing bacteremia. Virulence differences can obviously exist within strains of the same serotype, but it appears that certain serotypes of *P. aeruginosa* almost never cause bacteremic disease.

It is highly encouraging that bacteremic strains of *P. aeruginosa* have a limited serotype distribution and that antisera to the high-molecular-weight PS antigens follow the Fisher LPS typing system. The capability of immunization with high-molecular-weight PS to induce serotype-specific antibodies in rabbits, coupled with observations of the limited serotype diversity of bacteremic strains of *P. aeruginosa*, provides encouragement for the development of a high-molecular-weight PS vaccine that may be able to induce in humans the needed LPS type-specific immunity.

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Safety and Immunogenicity of High Molecular Weight Polysaccharide Vaccine from Immunotype 1 *Pseudomonas aeruginosa*

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ABSTRACT The safety and immunogenicity of a high molecular weight polysaccharide from immunotype 1 *Pseudomonas aeruginosa* were tested in a dose response fashion in adult volunteers. The vaccine lacked toxicity and pyrogenicity for experimental animals. Doses of 50, 75, 150, or 250 μg were given to groups of individuals as a single dose subcutaneous injection. Doses of 150 and 250 μg were associated with a significant rise in binding and opsonic antibody at 2 wk postimmunization. Titers remained unchanged for up to 6 mo. The vaccine was almost devoid of toxicity, eliciting no more than a slightly sore and tender arm at the site of injection. High molecular weight polysaccharide antigen appears to induce a good immune response following vaccination that is effective in mediating opsonophagocytic killing of live *P. aeruginosa* organisms.

INTRODUCTION

Infections caused by *Pseudomonas aeruginosa* have been particularly difficult to treat due to the organism's resistance to many antibiotics, the severity of the hosts' underlying condition that predisposes to *P. aeruginosa* infection, and the rapidity with which a septicemia can be fatal (1, 2). Immunotherapeutic modalities have been proposed as a potential means of increasing host resistance to this organism. Antibody directed towards cell surface lipopolysaccharide (LPS)[1] determinants has been shown to be effective in mediating opsonophagocytic killing of *P. aeruginosa* (3, 4). This antibody has been detected in the serum of patients convalescing from *P. aeruginosa* sepsis (5, 6), and survival of a *P. aeruginosa* sepsis episode has been associated with high levels of antibody to LPS in the acute phase serum (7). Attempts to induce antibody to LPS determinants in burn patients (8), cancer patients (9), and children with cystic fibrosis (10) have been hampered by the toxicity of LPS when used as a human vaccine. Nonetheless, these studies did suggest a drop in *P. aeruginosa* associated mortality following the use of an LPS vaccine. Recently, Jones et al. (11) documented the efficacy of a *P. aeruginosa* vaccine plus immunoglobulin in burn patients. Although the serologically active component of this vaccine has yet to be identified, the method of preparation (12) suggests it may be LPS.

A safe and immunogenic vaccine containing *P. aeruginosa* LPS serotype determinants would thus appear to be an ideal candidate for an immunotherapeutic agent to prevent *P. aeruginosa* sepsis. High molecular weight polysaccharide (PS) isolated from the supernate of *P. aeruginosa* cultures has been shown to be immunogenic in animals (13), to elicit protection to live organism challenge (14, 15) and to be nontoxic in mice and guinea pigs and nonpyrogenic in rabbits (14, 15). These PS antigens share serological specificity with the "O" specific side chain of LPS, yet differ from "O" side chains by their immunogenicity, biochemical constituents, monosaccharide composition, and molecular size (14, 15). Intact LPS contains the toxic lipid A component that is lacking in PS. Rabbit antisera to PS antigens contains antibody primarily directed at the LPS "O" side chain determinant, yet lacks anitbody to a second LPS-specific determinant present on the LPS molecule from all of the seven Fisher immunotypes of *P. aeruginosa*.[2] Thus animal

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[1] *Abbreviations used in this paper*: IT-1, immunotype 1; LPS, lipopolysaccharide.

[2] Pier, G. B., and D. M. Thomas. 1981. High molecular weight polysaccharide serotypes of *Pseudomonas aeruginosa*. Submitted for publication.

studies have shown that PS is capable of inducing an antibody response directed at LPS serotype determinants, yet lacks the toxicity associated with LPS vaccines. The present study was designed to assess the immunogenicity and safety of a prototype PS vaccine isolated from the immunotype 1 (IT-1) strain of *P. aeruginosa* in adult human volunteers, including an assessment of the functional nature of the antibody induced.

METHODS

Vaccine. High molecular weight PS antigen was extracted from a 30-liter culture of IT-1 *P. aeruginosa* grown in trypticase soy broth (Difco Laboratories, Detroit, Mich.) supplemented with 1% sodium acetate. After 48 h of growth, 300 g of hexadecyltrimethylammonium bromide was added and the precipitate and the organisms removed by centrifugation. The supernate was then concentrated to 800 ml on an Amicon TCE 5 ultrafiltration apparatus using PM 30 membranes (Amicon Corp., Danvers, Mass.). The crude PS-containing material was precipitated from the concentrate by the addition of 4 vol of 95% ethanol and recovered by centrifugation. This material was redissolved in phosphate-buffered saline (0.1 M phosphate, 0.15 M NaCl, pH 7.2) heated at 60°C for 1 h, and a one-tenth volume of 10% hexadecyltrimethylammonium bromide added to precipitate nucleic acids. After centrifugation, the supernate was recovered, crude PS precipitated by the addition of 4 vol of 95% ethanol, and the above procedure for removing nucleic acids repeated twice. Following this, the crude PS was dissolved in 1% acetic acid, the pH adjusted to 5.0 with glacial acetic acid, and the solution heated at 90°C for 18 h. This procedure cleaved the LPS into its lipid A and "O" side chain components for subsequent removal. After cooling, the lipid A precipitate was removed by centrifugation, the supernate extracted 10 times with chloroform then twice with 90% phenol, precipitated with 4 vol of 95% ethanol, redissolved in PBS, and applied to a Sephacyl S-300 column 2.6 × 100 cm in four separate runs. The serologically active material eluting between the void volume and the point where a 70,000-mol wt dextran marker begins to elute was collected, precipitated with alcohol, recovered, dialyzed, and lyophilized. This material was then weighed, dissolved to 1 mg/ml in pyrogen-free water with 1:30,000 merthiolate added and lyophilized as 1-mg aliquots in individual vials. Sterility of the material was ascertained in bulk before packaging and in 10% of the final packaged material in accordance with the Food and Drug Administration regulations (Title 21, Sect. 610.12). Prior to injection the vaccines were reconstituted with an appropriate amount of sterile saline for injection to give the desired dosage in 0.5 ml.

Chemical analyses. Analyses for nucleic acids, proteins, LPS, lipids, phosphate, carbohydrate, monosaccharide components, and water were performed as described (13).

Animal toxicity studies. The general safety test using guinea pigs (Title 21, Section 610.11) was done in two Hartley strain animals weighing 325 and 345 g. The animals were given 500 μg of PS in 5 ml saline, observed and weighed daily. The growth rate of 21 g mice was observed following intraperitoneal injection of 500 μg PS in 0.5 ml saline. Pyrogenicity was tested in three New Zealand White rabbits weighing between 2.04 and 2.50 kg following intravenous injection of 300 μg/kg body wt. Rectal temperatures were recorded prior to immunization and hourly for 3 h thereafter. Endotoxin contamination was tested for by the limulus lysate coagulation method (Sigma Chemical Co., St. Louis, Mo.). Two 12-kg rheuses monkeys were given four injections of 100 μg of PS subcutaneously at 3-d intervals and observed for local and systemic reactions for 72 h after each injection. Sera were collected before injection and weekly for 4 wk following the final injection.

Subjects. 42 normal healthy adult volunteers were asked to participate in this study. Signed informed consent was obtained, the volunteers randomly assigned to one of four groups receiving various doses of the vaccine, 20 ml of blood obtained by venipuncture, then a 0.5-ml subcutaneous injection of the vaccine given in the deltoid region of the arm. Subjects were interviewed at 24 and 48 h after the injection, symptoms noted, and temperatures recorded. Postimmunization sera were obtained at 14- and 28-d intervals following injection. For some subjects, serum was also obtained 6 mo postinjection.

Serologic methods. Serum antibody levels to the IT-1 PS were quantitated by means of a radioactive antigen binding assay using intrinsically labeled [^{14}C]PS prepared as previously described (13, 16). Sera were separated and stored at −20°C. Quantitation was performed as previously described for animal sera (16) except that five human sera were used to establish a standard curve. The correlation coefficient between percentage binding in the radioactive antigen binding assay vs. $\log_{10}$ microgram per milliliter of antibody was 0.901.

Opsonophagocytosis assays were performed by an adaptation of the methods of Baltimore et al. (17) and Young (4). Human peripheral blood leukocytes were purified on a dextran gradient, freed of erythrocytes, and suspended to 10^7 cells per milliliter. *P. aeruginosa* IT-1 was harvested in mid-log growth phase, washed once with minimal essential media (Microbiological Associates, Bethesda, Md.) and resuspended to 3×10^7 organisms/ml. The reaction mixture consisted of 100 μl of the serum or serum dilution to be tested, 100 μl of cells, 100 μl of organisms, and 100 μl of a 1:5 dilution of guinea pig complement. A 25-μl aliquot was removed from the tube at time 0, diluted in distilled water to lyse the leukocytes, then further diluted in saline and plated out on trypticase soy agar plates for bacterial enumeration. A similar aliquot was removed following 60 min of incubation at 37°C where tubes were continuously mixed, and organisms counted. The opsonic titer of the serum was expressed as the reciprocal of the serum dilution killing 90% or more of the initial inoculum. Controls for each experiment included mixtures of two of three components (cells, serum, and complement) plus organisms and media.

Statistical methods. Differences in the concentration of antibody in preimmunization and postimmunization sera were compared by a *t* test (18). Antibody titer rises of fourfold or greater in the opsonophagocytic assay were considered a positive response and analysis of responses between groups receiving different doses were compared by logistic regression (19).

RESULTS

Chemical analyses. The results of analyses for the various biochemical and monosaccharide constituents of the IT-1 high molecular weight PS are shown in

TABLE I
Chemical Analyses of the P. aeruginosa IT-1 Vaccine

	%
Component	
Carbohydrate (Total)	72.5
Lipid	<0.5
Phosphate	<0.5
Nucleic acid	0.8
Protein	0.9
Water	22.2
Monosaccharide constituents	
Arabinose	6.1
Rhamnose	3.4
Mannose	62.2
Galactose	19.8
Glucose	8.5

Table I. Consistent with previously published results (13) the antigen was composed principally of carbohydrate, with low levels of contaminating nucleic acids, protein, and LPS. A high level of mannose was also found along with the previously reported monosaccharides of arabinose, rhamnose, galactose, and glucose (13).

Animal studies. The results of animal tests and in vitro coagulation of the limilus amebocyte lysate for toxicity indicated that the PS vaccine passed these tests with no indication of toxicity. These tests were performed on final packed material rehydrated with sterile saline for injection. No significant rises in temperature (<0.5°F) were detected in rabbits given 300 μg/kg body wt. Further lack of biologically active endotoxins was seen in the limulus lysate assay, where it took 1,000 times more vaccine than control LPS to gel the lysate (Table II). General toxicity tests in guinea pigs, mice, and monkeys revealed normal weight gains following injection of up to 500 μg PS vaccine. The two monkeys given four 100-μg injections developed both binding antibody and titer rises of fourfold or greater by opsonophagocytosis.

Toxicity in human volunteers. Four different doses were given to volunteers: 7 persons received 50 μg, 5 persons received 75 μg, 12 persons received 150 μg, and 18 persons received 250 μg. Reactions to the PS vaccine were exceedingly mild, and no greater reaction than soreness and slight tenderness at the injection site were noted for any vaccinee at any dose, except for one person receiving 150 μg who was scratched by the needle under the injection site and developed a slightly red and tender area lasting for 48 h. No reaction lasted >48 h, and no erythema or induration was seen in any vaccinee other than as noted above. Slight soreness and tenderness at the injection site was seen in 1 of 7 (14.3%) persons given 50 μg, 1 of 5 (20%) persons given 75 μg, 4 of 12 (33.3%) persons given 150 μg, and 12 of 18 (66.7%) persons given 250 μg.

Antibody response. The antibody responses of subjects in each of the four dosage groups 2 and 4 wk postimmunization, as quantitated in the radioactive antigen binding assay, are shown in Table III. There was no significant difference between preimmunization and postimmunization concentrations of antibody in the group given 50 of 75 μg (*t* test). In the group given 150 μg a significant ($P = 0.004$) difference in the mean pre- and postimmunization antibody concentrations were noted. Similarly, at 250 μg a significant ($P = 0.002$) difference in antibody concentration was

TABLE II
Analyses for Endotoxin Contamination by Gelation of the Limulus Amebocyte Lysate

	Component		
Amount	IT-1 PS vaccine	IT-1 LPS	*Escherichia coli* LPS standard
ng			
0.1	–*	–	–
0.5	–	–	+
1.0	–	+	+
10.0	–	+	+
100.0	–	+	+
1,000.0	+	+	+

* + Indicates gelation of lysate; – indicates no gelation after 24 h.

TABLE III
Immunogenicity of PS Vaccine from IT-1 P. aeruginosa

	Antibody concentration geometric mean±SD (range), μg/ml		
	Weeks after immunization		
Dose	0	2	4
μg			
50	13.2±12.8 (3.9–40.0)	30.5±29.2 (4.0–88.4)	31.2±30.3 (4.0–86.8)
75	22.8±41.9 (4.1–97.8)	42.8±73.9 (3.8–174.3)	44.5±76.8 (3.8–175.2)
150	5.9±3.6 (1.7–14.9)	63.8±55.8 (6.1–147.4)	66.7±54.3 (6.1–155.8)
250	4.9±1.9 (3.6–10.2)	55.9±61.8 (3.7–250)	56.3±59.3 (3.7–232)

TABLE IV
Duration of Antibody Levels in Persons Receiving 150 μg of IT-1 P. aeruginosa PS Vaccine

Antibody concentration in μg/ml-geometric mean±SD (range)		
Time after immunization		
0	6 mo	Difference (post-pre)
5.9±3.6 (1.7–14.9)	32.7±29.3 (4.2–94.2)	27.3±28.8 (1.1–87.4)

noted. No significant difference was noted in the geometric mean antibody titer achieved at 2 wk when compared to 4 wk by a pooled *t* test, and no significant difference was noted between the geometric mean antibody concentration achieved in the sera of vaccinees receiving 150 μg (63.8 μg/ml) vs. that achieved at the 250-μg dosage (55.9 μg/ml). Serum antibody levels present at 6 mo following immunization with 150 μg are shown in Table IV. The decrease in individual antibody levels was not significant, indicating maintenance of antibody titers during this interval.

Functional properties of the induced antibodies were measured in an opsonophagocytosis test, and the titers determined for pre- and day 14 postimmunization sera are shown in Table V. 3 of 7 persons receiving 50 μg had a fourfold or greater increase in titer in this assay, while 2 of 5 persons receiving 75 μg, 9 of 12 persons receiving 150 μg, and 16 of 18 persons receiving 250 μg had these responses. In the total population, 32 persons had preimmunization titers of two or less, 8 had preimmunization titers of four through eight and 2 had preimmunization titers of more than eight. After immunization, 7 persons had titers of 2 or less, 13 had titers of 4–8 and 23 had titers of 1:16 or greater, up to 1:128. Logistic regression analyses of the dose response effect was performed on these data. Responses were designated 1 or 0 to indicate whether or not a fourfold or greater titer rise had occurred. The responses at 50 and 75 μg were treated as one category (low dose) for these statistical purposes. A highly significant difference ($P < 0.001$) was observed between the response seen in the group immunized with 150 and 250 μg, when compared to the response of the 50- and 75-μg group. The difference in response between 150- and 250-μg doses was marginally significant, ($P = 0.079$). This suggested a trend for the higher dose being slightly more efficacious in inducing a functional antibody response.

DISCUSSION

Disease due to *P. aeruginosa* infections is most often seen in immunocompromised or traumatized hosts. Susceptibility to infection has been thought to be correlated with granulocytopenia (2), though the underlying host condition was found to be a better indicator of the severity and outcome of *P. aeruginosa* sepsis (20). These immunocompromised patients, who are at risk for developing *P. aeruginosa* infections, are altered in their responses to immunological stimuli, and therefore may not respond to the PS antigen with humoral antibody, as was shown here for normal volunteers. Vaccination of granulocytopenic populations generally results in poor immune responses, but there are certain populations who are at high risk for *P. aeruginosa* infections that can be immunized prophylactically. Other populations, such as burn and trauma victims, may respond adequately to vaccination if given soon enough after injury. Immunosuppressed populations can potentially be immunized before or in between courses of therapy. Since the PS vaccine used here induced both binding and opsonic antibody, and has minimal toxicity in vaccinees, it offers the possibility to function as an effective immunotherapeutic agent for preventing *P. aeruginosa* sepsis.

TABLE V
Serum Titers in the Opsonophagocytosis Assay following Immunization with P. aeruginosa IT-1 PS Vaccine

Dose	Number immunized	Preimmunization titer			Postimmunization titer			No. persons with fourfold or greater rise
		≤2	4–8	>8	≤2	4–8	>8	
μg								
50	7	5*	2	0	3	2	2	3
75	5	4	1	0	3	1	1	2
150	12	9	1	2	1	5	6	9
250	18	14	4	0	0	5	13	16

* Represents number of vaccinees with this titer.

PS antigens are prepared by a method utilizing heat and acid to cleave the contaminating LPS into its lipid A, "O" side chain and core components for subsequent elimination. This somewhat harsh method was chosen because it was found to be the only method that removed all detectable intact LPS. Although low levels of LPS contamination in a vaccine may not be of any concern if the toxicity is within acceptable limits, animal studies of numerous *P. aeruginosa* vaccines have often indicated that contaminating LPS is the responsible agent for the immunogenicity and protective efficacy seen (14). The immunogenicity of this PS vaccine in humans, coupled with its almost total lack of toxicity, indicates that the acetic acid method for elimination of LPS is not only an effective procedure for reducing toxicity, but does not interfere with immunogenicity.

The magnitude of the human immune response to PS is particularly good when compared to the amount of antibody inducible in experimental animals (16). Humans by far had a greater degree of response than we have found for the most responsive laboratory animal, the C_3H mouse strain. The opsonic titers we obtained in our vaccinees was also close to that reported by Young and Armstrong (3) and Young (4) for patients recovering from *P. aeruginosa* sepsis or immunized with an LPS vaccine. The opsonophagocytosis test they used was very similar to the one used here, except that they multiplied their titers by a factor of 10 to translate the 0.1-ml amount of serum used in the reaction mixture to 1.0 ml. The data here report the dilution of a 0.1-ml amount of serum that elicited killing. Another slight difference was their use of ≥70% reduction in viable organisms as representing killing, while we used ≥90% levels. Taking this into account, the phagocytic titers of 12 persons recuperating from *P. aeruginosa* bacteremia ranged from 32 to 2,048 (our method), with 8 of these patients (66.7%) having titers of 32–128. 12 of 18 (66.7%) of the PS vaccinees receiving 250 μg had titers of 16–128. None of the immunized individuals had a titer of >128, whereas 4 of 12 of the infected patients did. Similarly in Young's (4) study of the opsonic titers of humans given monovalent preparations of *P. aeruginosa* LPS, opsonic titers of 256–2,048 were obtained. Although these titers are slightly higher than those obtained by PS vaccination, the dose of LPS used was quite high when compared to PS (25 μg/kg LPS vs. an average of 3.5 μg/kg PS) was given in five doses when compared with the single dose of PS, and was associated with local reactions not seen with PS immunization.

The influence of antibody levels of *P. aeruginosa* LPS serotype determinants in affecting the outcome of *P. aeruginosa* infection has been suggested by both vaccine studies (8, 9, 11) and also by a study of the influence on survival of acute phase antibody levels to LPS (7). Our studies in animals, (14, 15) indicate that PS induces a serotype specific immune response against the LPS "O" side chain determinant. Further study is required to assess whether the human immune response to PS vaccination also induces a response to LPS specific determinants. The data here do indicate that immunization with PS leads to an immune response in humans, that the antibody elicited can function in opsonizing live organisms, and that PS vaccination is associated with a minimal level of toxicity in vaccinees.

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INDUCTION IN MICE OF CELL-MEDIATED IMMUNITY TO *PSEUDOMONAS AERUGINOSA* BY HIGH MOLECULAR WEIGHT POLYSACCHARIDE AND VINBLASTINE[1]

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The effect of the cytotoxic drug vinblastine on the development of immunity to high m.w. polysaccharide (PS) isolated from culture supernates of *Pseudomonas aeruginosa* was investigated. One microgram of PS, a normally nonimmunogenic, nonprotective dose, plus 75 μg of vinblastine were administered to BALB/c mice, and afforded protection to live organism challenge with the homologous strain. The kinetics and serotype specificity of the immune response indicated an active immunization had occurred. Analyses of serum antibody levels of mice given the PS-drug regimen in a sensitive, radioactive antigen-binding assay (RABA) failed to show development of antibody to the immunizing PS. Immunity could be passively transferred with spleen cells but not by serum from PS-drug-immunized animals, and the effector cell was removed by antisera to the Thy-1.2 antigen. Nu/nu mice were also protected against challenge after immunization with PS and vinblastine, but this protection was observed in association with the development of serum antibody to PS in these mice, as measured in the RABA. Protective immunity could not be elicited in the BALB/c mice by PS plus cyclophosphamide. These data suggest that under certain conditions, PS antigens can elicit T cell-dependent immune phenomena, and this T cell-dependent immunity can protect mice from live organism challenge against an extracellular bacterial pathogen.

Immune protection against extracellular bacterial pathogens has been well correlated with the development of antibodies specific for antigens present on bacterial surfaces (1). The ability of antibodies to promote phagocytosis and killing of extracellular bacteria *in vitro* (2) and the protection afforded to nonimmune recipients by passively transferred immune sera (3) demonstrated the critical role antibodies play in immunity against such organisms. It has also been amply documented that killing of those bacteria, such as *Listeria monocytogenes*, that live intracellularly and are inaccessible to the effects of antibodies is critically dependent on the function of effector T cells (4). Antibody provides minimal if any protection against such organisms (2, 4).

The possibility that T cells might also play a role in immunity to extracellular bacteria has not been extensively explored. Difficulty in demonstrating a role for effector T cells in situations in which antibody-mediated protection is so efficient, probably accounts, in part, for the paucity of data on the role of cell-mediated immunity (CMI)[3] in relation to extracellular bacterial infections. Recent studies of protection induced in mice by immunization with a high m.w. polysaccharide (PS) antigen isolated from culture supernates of the extracellular bacterial pathogen, *Pseudomonas aeruginosa* immunotype (IT-1) (5), demonstrated the ability of this PS to induce antibodies that protect mice from live bacterial challenge (6, 7). During the course of these protection studies, it was discovered that BALB/c mice generated a protective antibody response to a 50-μg dose of the IT-1 PS, but failed to produce antibodies after immunization with a limiting 1 μg dose of IT-1 PS. Mice were also not protected from subsequent live bacterial challenge after the low dose immunization. It was observed, however, that if BALB/c mice received the mitotic inhibitor vinblastine sulfate, at the time of immunization with 1 μg of the IT-1 PS, they were protected against bacterial challenge, despite the fact they still failed to produce an antibody response. The present report provides evidence that the protection against *P. aeruginosa* infection observed in BALB/c mice receiving vinblastine plus 1 μg of IT-1 PS is T cell-mediated.

MATERIALS AND METHODS

Immunogens. High m.w. PS from *P. aeruginosa* IT-1 was prepared as previously described (5). Vinblastine sulfate (Velban) was obtained from Eli Lilly and Co., Indianapolis, IN. Cyclophosphamide (Cytoxan) was obtained from Meade Johnson Pharmaceutical Co., Evansville, IN.

Bacteria. Strains of *P. aeruginosa* IT 1–7 were kindly provided by Dr. M. Fisher, Parke-Davis Co., Detroit, MI.

Mice. Eight-week-old BALB/c mice were obtained from Cumberland View Farms, Clinton, TN. Nu/nu and Nu/+ littermate BALB/c mice were obtained from Charles River Breeders, Wilmington, MA.

Immunization and challenge protocol. Mice were immunized with the indicated dosage of IT-1 PS in 0.5 ml of phosphate-buffered saline (PBS, pH 7.2). The PBS used had to be passed through a DM-5 ultrafiltration membrane (Amicon Corp., Danvers, MA) to eliminate a factor that gave nonspecific protection with vinblastine alone. The vinblastine was given as a 75-μg dose in 0.2 ml ultrafiltered PBS. Cyclophosphamide was administered in 0.2 ml ultrafiltered PBS i.v. 48 hr before antigen. The mice were challenged at the indicated time interval after immunization with live *P. aeruginosa* organisms in 0.1 ml saline i.p. The challenge dose for *P. aeruginosa* IT-1, 2, and 4 was 10^6 organisms per mouse; for *P. aeruginosa* IT-5, 6, and 7, 10^7 organisms per mouse; and for *P. aeruginosa* IT-3, 5×10^6 organisms per mouse. The challenge doses were prepared from overnight cultures grown on trypticase soy agar, suspended in saline, standardized by optical density measurements at 650 nm, then administered as a 0.1 ml i.p. dose. Deaths were recorded for 96 hr after challenge.

Antisera. Murine sera were obtained via retroorbital bleeding of ether-anesthetized animals.

Serologic assay. Concentrations of antibody in micrograms per milliliter were determined by use of a radioactive antigen-binding assay (RABA) as

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[3] Abbreviations used in this paper: PS, polysaccharide; IT, immunotype; RABA, radioactive antigen-binding assay; CMI, cell-mediated immunity; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MIF, macrophage inhibition factor.

described (7, 8). The antigen used was intrinsically labeled ^{14}C PS prepared as described (7, 8). The lower limit of detection of antibody in this assay for mouse antibodies is 2 μg/ml.

Passive transfer studies. Passive transfer of serum was done by giving the indicated amount of mouse antiserum i.p. 3 hr before challenge with 10^6 live *P. aeruginosa* IT-1 organisms. Passive transfer of spleen cells was performed by transferring the indicated number of cells in 0.5 ml Hanks' balanced salt solution (HBSS, Microbiological Associates, Bethesda, MD) via an i.p. injection. Cells were obtained from the spleens of animals by passing the intact spleens through a wire mesh screen to disperse the cells, then removing large fragments by passing the cell suspension through a funnel loosely packed with glass wool. The cells in this suspension were pelleted by centrifugation and resuspended in HBSS, viable cell counts were determined by trypan blue dye exclusion, and the cell concentration was adjusted to the desired amount. Challenge of recipient mice was 72 hr later with 10^6 live *P. aeruginosa* IT-1 cells.

Selection of lymphocyte subpopulations. T cells and B cells were selectively removed from spleen cell suspensions by incubation of 3×10^7 cells in 3 ml HBSS on petri plates coated with either mouse anti-Thy-1.2 sera, rabbit antisera to mouse immunoglobulin (Ig, Accurate Chemical Co., Hicksville, NY), or normal mouse serum (NMS) for 45 min at 4°C (9). The nonadherent cells were removed, were pelleted by centrifugation, and were resuspended in the volume of HBSS that gave 2×10^7 cells before the depletion step.

Determination of efficacy of lymphocyte subpopulation separations. After depletion of either T cells or B cells, an aliquot of the cell suspension was taken and the cells were pelleted by centrifugation and resuspended in RPMI 1640 medium containing HEPES[4] buffer, L-glutamine, penicillin, and streptomycin. The volume was chosen to give 10^7 cells/ml before depletion. Then, 100 μl (10^6 cells) of this cell suspension were placed into round-bottomed microtiter wells and the following mitogens were added to triplicate cultures: phytohemagglutinin (PHA), 2 μg/ml; concanavalin A (Con A), 0.2 μg/ml; and lipopolysaccharide (LPS), 5.0 μg/ml, from *Escherichia coli* 0:26 (Difco Laboratories, Detroit, MI). Cultures were then assayed for mitogenic stimulation as described (8).

Statistics. P values for survival from live cell challenge were calculated by a Fisher Exact Test. P values for differences in pre- and post-immunization serum antibody levels were calculated by a t-test.

RESULTS

Protection of BALB/c mice by immunization with IT-1 PS plus vinblastine. Groups of 10 mice were immunized with either 1 μg of IT-1 PS plus 75 μg vinblastine, 1 μg of IT-1 PS alone, 75 μg of vinblastine alone, or ultrafiltered PBS, and were challenged 7 days later with 10^6 live *P. aeruginosa* IT-1 cells i.p. The data (Table I) show that the mice given 1 μg IT-1 PS i.p. plus 75 μg vinblastine i.v. (within 5 min of each other) were significantly ($p < 0.001$, Fisher Exact Test) protected from challenge with live *P. aeruginosa* IT-1 cells. No protection was evident among mice given the PS or drug alone, or mice given PBS.

Immunotype specificity of PS plus vinblastine-induced protection. To determine if the protection observed was specific, four groups of 70 mice each were immunized with either 1 μg of the IT-1 PS plus 75 μg of vinblastine, 1 μg of the IT-1 PS only, 75 μg of vinblastine only, or PBS. Within each group of 70, subgroups of 10 mice were challenged 7 days later with the indicated doses of live *P. aeruginosa* cells from each of the seven Fisher immunotypes. Protection was only seen against challenge with the homologous IT-1 strain of *P. aeruginosa* in the mice immunized with 1 μg IT-1 PS plus 75 μg of vinblastine (Table II), indicating the serotype specificity of the immunity elicited.

Kinetics of induction of protective immunity. Kinetics of the immunity induced by immunization with the PS-drug protocol was then examined. Five days after immunization, 40% protection was noted (Table III), which increased to 100% by the sixth day after immunization. The protective level fell to 40% by day 17 post-immunization. Attempts to boost the duration of immune protection by giving three doses of the PS-drug regimen at 7-day intervals indicated 80 to 100% protection 35 days after the last injection (Table IV). The immunity began to wane after this time, but significant protection was still observed 49 days after the final dose (Table IV).

The effect of varying the time interval between PS immunization and vinblastine administration was next studied. Vinblastine given from 72 hr before until 8 hr after PS was effective in eliciting protection from live organism challenge (Table V).

TABLE I

Protection of BALB/c mice to live organism challenge after immunization with high m.w. PS from P. aeruginosa IT-1 and vinblastine

Immunized with:	Dose	No. Mice per Group	Number Survivors	Percentage Survivors	P Value
	μg				
High m.w. PS plus Vinblastine	1 75	10[a]	8	80	<0.001
High m.w. PS	1	10	0	0	
Vinblastine	75	10	2	20	0.24
PBS		10	0	0	

[a] These mice bled for serum antibody studies in Table VI.

TABLE II

Serotype specificity of protection elicited in BALB/c mice given 1 μg IT-1 high m.w. PS plus 75 μg vinblastine

Serotype of Challenge Strain	PS[a] + Vin	Vin Only	PS Only	PBS Only	P Value PS + Vin vs PBS only
IT-1	10^6/10	0/10	1/10	0/10	<0.001
IT-2	2/10	1/10	0/10	0/10	0.24
IT-3	2/10	2/10	1/10	0/10	0.24
IT-4	0/10	1/10	1/10	0/10	1.0
IT-5	0/10	0/10	0/10	0/10	1.0
IT-6	0/10	0/10	1/10	0/10	1.0
IT-7	1/10	0/10	0/10	0/10	0.5

[a] PS, high m.w. polysaccharide from IT-1 *P. aeruginosa*; Vin, vinblastine.
[b] Represents number survivors over total challenged. Mice immunized with PS + Vin and challenged with IT-1 cells were bled for serum antibody study in Table VI.

TABLE III

Kinetics of protection of BALB/c mice after immunization with 1 μg IT-1 high m.w. PS and 75 μg vinblastine

Days between immunization and Challenge	Immunized with: PS[a] + VIN	VIN only	PS only	PBS only	P Value PS+Vin vs PBS
24	3[b]/10	1/10	0/10	0/10	0.105
21	5/10	1/10	0/10	0/10	0.016
17	4/10	0/10	0/10	0/10	0.043
14	8/10	0/10	1/10	0/10	<0.001
10	9/10	0/10	1/10	0/10	<0.001
7	10[c]/10	1/10	1/10	0/10	<0.001
6	10/10	1/10	0/10	0/10	<0.001
5	4/10	0/10	1/10	0/10	0.043
4	0/10	0/10	0/10	0/10	1.0
3	0/10	1/10	1/10	0/10	1.0
2	0/10	0/10	0/10	0/10	1.0
1	0/10	1/10	0/10	0/10	1.0

[a] PS, high m.w. polysaccharide from IT-1 *P. aeruginosa*; Vin, vinblastine.
[b] Number survivors over total challenged.
[c] These mice bled for serum antibody determinations in Table VI.

TABLE IV

Increase in duration of immunity in BALB/c mice given 3 doses of 1 μg IT-1 PS plus 75 μg vinblastine at 7-day intervals

Days after Final Injection	Immunized with: PS + Vin	Vin only	PS only	PBS only	P Value, PS + Vin vs PBS
17	10[a]/10	1/10	0/10	0/10	0.001
49	4/10	0/10	1/10	0/10	0.043
45	6/10				
42	6/10				
35	10/10				
31	10/10				
24	8/10				
21	10/10				

[a] Number of survivors over total challenged. Mice challenged 17 days after the final injection were bled for serum antibody determinations in Table VI.

had 16% of the PHA responsiveness, and 12% of the Con A responsiveness of NMS treated cell populations, while retaining 84% of their LPS responsiveness (Table IX). Anti-Ig-depleted cell populations retained 66% of their PHA and 84% of their Con A response, while the LPS-induced mitogenesis was reduced to 19% of NMS treated controls.

Protection in nu/nu BALB/c mice. We then looked at the ability of 1 μg of IT-1 PS plus 75 μg of vinblastine to protect nu/nu and nu/+ littermate control mice from live organism challenge 7 days after immunization. As expected, neither nu/nu or nu/+ mice were protected after a dose of 1 μg IT-1 PS alone or 75 μg of vinblastine alone (Table X). Unexpectedly, nu/nu mice were protected from live cell challenge after 1 μg of IT-1 PS plus 75 μg of vinblastine, as were the control nu/+ mice. However, when serum antibody levels (difference in post and preimmunization levels) of these mice were measured in the RABA, the nu/nu mice that received the PS-drug immunization regimen were found to have a significant ($p < 0.05$) increase in serum antibody levels to the IT-1 PS, whereas the nu/+ mice, similarly immunized, lacked detectable antibody (Table X). The antibody levels found in the nu/nu mice given the PS-drug immunization regimen were slightly higher than the antibody levels measured in the serum of nu/nu and nu/+ mice given an immunogenic 50-μg dose of the IT-1 PS. These data also showed that a response to the 50-μg dose of IT-1 PS could be generated in the absence of helper T cells.

Ability to induce protective immunity with IT-1 PS plus cyclophosphamide. Other investigators (11) demonstrated the ability of another immunopharmacologic agent, cyclophosphamide, to promote the development of CMI. To determine if the effects we observed with vinblastine could be duplicated by cyclophosphamide treatment, we immunized BALB/c mice with a wide range of doses of cyclophosphamide 48 hr before immunization with 1 μg IT-1 PS. We were unable to obtain protection to live organism challenge 7 days after the PS immunization at any of the doses of cyclophosphamide (Table XI). Thus, cyclophosphamide, over a wide dose range, was unable to duplicate the phenomenon observed with vinblastine, even though the conditions of cyclophosphamide administration were identical to those found to promote the development of CMI to cellular or protein antigens in other studies (11).

TABLE IX

Ability of spleen cell subpopulations to passively transfer immunity when taken from mice given 1 μg IT-1 high m.w. PS and 75 μg vinblastine

Cells[a] Treated on Plates Coated with:	SI[b] of Mitogens			No. Survivors Total recipients	P Value vs. Nonimmune Control
	PHA	Con A	LPS		
NMS	17.3	14.5	9.6	8/10	<0.001
Anti-Thy-1.2	1.4	1.7	8.2	2/10	0.24
Anti-Ig	11.4	12.2	1.9	9/10	<0.001
Nonimmune cells (control)	18.1	12.2	10.1	0/10	

[a] Originally 3×10^7 cells in 3 ml were placed on plates. After recovery cells resuspended in 1.5 ml HBSS and 0.5 ml given to recipient mice.

[b] SI, stimulation index, $\frac{\text{CPM, mitogen-stimulated cultures}}{\text{CPM, unstimulated cultures}}$.

TABLE X

Ability of 1 μg high m.w. PS plus 75 μg vinblastine to elicit protective immunity in nu/nu and nu/+ BALB/c mice

Immunized with	nu/nu Mice	Antibody Level (μg/ml)[a]	nu/+ Mice	Antibody Level (μg/ml)
50 μg PS	5[b]/5	19.8 ± 5.9	5[b]/5	21.2 ± 6.3
1 μg PS + 75 μg Vin	4/5	23.4 ± 8.1	4/5	<2.0
1 μg PS	1/5	<2.0	0/5	<2.0
75 μg Vin	0/5	<2.0	0/5	<2.0
PBS	0/5	<2.0	0/5	<2.0

[a] μg/ml antibody level determined in RABA assay.

[b] Represents number survivors over total challenged.

TABLE XI

Inability of cyclophosphamide plus 1 μg IT-1 high m.w. PS to induce immunity in BALB/c mice to live organism challenge

Dose (mg/mouse) Cyclophosphamide[a]	No. Survivors Total Immunized	P Value vs. PBS Control
0.0 (PBS control)	0/10	
0.4	2/10	0.24
1.0	0/10	1.0
2.0	0/10	1.0
3.0	2/10	0.24
4.0	0/10	1.0
5.0	0/10	1.0
PS only (1 μg)	0/10	1.0

[a] Cyclophosphamide administered I.v. via the tail vein in 0.2 ml ultrafiltered PBS 48 hr before a 1 μg I.p. dose of high m.w. PS in 0.5 ml ultrafiltered PBS.

DISCUSSION

The role of T cell-mediated immunity to infectious organisms is thought to be generally directed toward intracellular facultative parasites (4). Reports on the ability of activated macrophages to kill *Staphylococcus aureus* (12) and *Streptococcus pneumoniae* (13), as well as a role for T cell-dependent immunity in preventing abscess formation caused by *Bacteroides fragilis* (14), suggested extracellular bacterial pathogens can be killed by cellular immune mechanisms. Efforts to demonstrate the importance of T cells in protection from extracellular bacterial pathogens have been hampered by the efficiency of antibody-mediated effector mechanisms for protection against these organisms. In the studies reported here, we were able to induce specific protection against the extracellular bacterial pathogen, *P. aeruginosa*, IT-1, without stimulating antibody production. The protection observed could be adoptively transferred with immune spleen cells but not with serum from PS-vinblastine-immunized mice, and the effector cells were removed from immune spleen cell populations by antisera directed at the Thy-1.2 antigen present on T lymphocytes. Further information in nu/nu mice regarding the importance of T cells in the protection induced with IT-1 PS plus vinblastine was impossible to obtain because this immunization protocol allowed the development of antibody in these T cell-deficient mice. The reasons for this are unclear, but the data suggests that vinblastine relieves suppression in a non-T cell subpopulation of immunoregulatory cells. Nonetheless, it appears that a T cell-dependent immune effector mechanism can protect mice from live *P. aeruginosa* cell challenge following an appropriate immunization protocol. However, these data do not indicate what the specific T cell-dependent effector mechanisms are.

Because polysaccharide antigens are thought to be poor inducers of CMI, there was the possibility that a protein contaminant of the IT-1 PS preparation was responsible for the immunity elicited. Analyses of the composition of the IT-1 PS preparation does not support this possibility. Protein contamination of the IT-1 PS preparation is low (less than 1.0%) (5), indicating that any active protein contaminant would need to be effective at a dose of less than 0.01 μg. The serotype specificity of the protection would necessitate that a protein contaminant have a one to one association with the serotype determinant present on the PS and LPS of *P. aeruginosa*, a highly unlikely possibility. Furthermore, the major outer membrane proteins from strains of *P. aeruginosa* (which would be the most likely protein contaminants) are highly conserved among the seven Fisher strains used here (15). We have also recently tested (unpublished observation) the ability of perio-

TABLE V

Effect on development of immunity by varying the time interval between administration of 75 μg vinblastine and 1 μg IT-1 high m.w. PS

Interval between Vinblastine and PS Administration	No. Survivors Total challenged	P Value (vs PBS only)
−96	0/10	1.0
−72	10/10	<0.001
−48	10/10	<0.001
−24	10/10	<0.001
0[a]	9/10	<0.001
+2	10/10	<0.001
+4	10/10	<0.001
+8	9/10	<0.001
+24	2/10	0.24
+36	1/10	0.5
Vin only	1/10	0.24
PS only	0/10	1.0
PBS only	0/10	

[a] These mice bled for serum antibody determination in Table VI.

TABLE VI

Serum antibody levels (μg/ml) of mice given 1 μg IT-1 high m.w. PS plus 75 μg vinblastine in experiments in Tables I to V

Expt. in Table:	No. Mice	Preimmune Avg. (range)[a]	Postimmune Avg. (range)	Difference of Avg. (μg/ml)	P Value[b]
I	10	2.6 (<2–4.1)	2.9 (<2–4.0)	0.1	NS[c]
II	10	2.7 (<2–3.9)	2.6 (<2–3.9)	−0.1	NS
III	10	2.6 (<2–3.3)	2.2 (<2–3.3)	−0.6	NS
IV	10	3.1 (<2–4.5)	3.3 (<2–4.6)	0.2	NS
V	10	2.6 (<2–3.9)	2.9 (<2–4.5)	0.3	NS
50 μg PS (control)	10	2.2 (<2–4.1)	25.4 (14.7–30.7)	23.2	<0.01

[a] Lower limit of test sensitivity for mouse serum is 2 μg/ml.
[b] Calculated by Student's *t*-test.
[c] NS, nonsignificant, $p > 0.05$.

Vinblastine given 96 hr before PS immunization, or 24 hr or more after PS, failed to induce protection.

Involvement of serum factors in PS-vinblastine-induced immunity. To understand the basis for the apparent immunity, serum samples taken from some of the mice in the above experiments (Tables I–V) 3 hr before challenge were analyzed for serum antibody to IT-1 PS in the RABA. The groups of mice tested are denoted by footnotes in Tables I–V. Positive controls for this test were sera obtained from BALB/c mice immunized with a 50-μg dose of IT-1 PS, which is known to stimulate antibody formation. Table VI shows that in none of 50 mice given 1 μg IT-1 PS plus 75 μg of vinblastine was there any evidence for an increase in serum antibody levels. Mice immunized with 50 μg of the IT-1 PS alone, however, showed good serum antibody rises.

To ascertain if another serum factor, not measurable in the RABA, was the responsible protective entity, we transferred serum from mice immunized 7 days previously with either 1 μg IT-1 PS alone, 50 μg IT-1 PS alone, 1 μg IT-1 PS plus 75 μg vinblastine, 75 μg vinblastine only, or NMS to nonimmune recipients. Mice were then challenged 3 hr after passive transfer of serum with about 10^6 live *P. aeruginosa* IT-1 cells. Only mice given 0.25 ml of serum from the group immunized with the immunogenic 50-μg dose of IT-1 PS were protected from live organism challenge (Table VII). Up to 0.6 ml of the other sera were not effective in transferring immunity. Above the 0.6-ml serum amount, all of the sera, including NMS, had protective efficacy. This phenomenon has been noted in passive transfer of nonimmune sera to recipient mice challenged with other Gram-negative organisms (10).

Role of immune spleen cells in transferring PS-drug-induced immunity. Because serum factors did not appear to be involved in the immunity elicited by the PS-vinblastine combination, we looked at the ability of spleen cells to transfer immunity. Table VIII shows that 10^7 cells from mice given the PS-drug regimen, when transferred to a recipient mouse, were effective in providing protection to live cell challenge 72 hr after adoptive cell transfer. No passive protection was noted when up to 10^8 cells taken from nonimmune animals or animals given either 1 μg IT-PS alone or 75 μg vinblastine alone, were transferred to recipients. No detectable antibody, as measured in the RABA, was found in the adoptively transferred recipient mice 3 hr before challenge (data not shown).

Identity of the effector cell transferring immunity. Immune spleen cell populations were selectively depleted of lymphocytes bearing either the Thy-1.2 determinant or surface Ig by treatment of 4°C on petri plates coated with either mouse anti-Thy-1.2, rabbit anti-mouse Ig, or NMS (9). The selected populations were then transferred to nonimmune recipient mice that were challenged 72 hr later with live *P. aeruginosa* IT-1 cells. The results (Table IX) indicated that the effector cell transferring immunity was removed by antisera directed at the Thy-1.2 determinant and not by antisera directed at the Ig determinants. Testing of these cell populations for mitogenic stimulation by the T cell mitogens PHA and Con A, and the B cell mitogen, LPS, showed the anti-Thy-1.2-treated cells only

TABLE VII

Passive protection of mice after transfer of mouse serum raised to either 50 μg IT-1 PS, 1 μg IT-1 PS plus 75 μg vinblastine, 1 μg IT-1 PS only, 75 μg vinblastine only or normal mouse sera (NMS)

Serum Given (raised to)	Amt	No. Survivors Total recipients	P Value vs NMS Control
	ml		
50 μg IT-1 PS	0.25	5/5	0.001
1 μg IT-1 PS + 75 μg Vinblastine	0.25	0/5	NS[a]
	0.50	0/5	NS
	0.60	2/5	NS
	0.70	4/5	NS
1 μg IT-1 PS only	0.25	0/5	NS
	0.50	1/5	NS
	0.60	4/5	NS
	0.70	5/5	NS
75 μg Vinblastine only	0.25	0/5	NS
	0.50	1/5	NS
	0.60	3/5	NS
	0.70	4/5	NS
NMS	0.25	0/5	
	0.50	0/5	
	0.60	3/5	
	0.70	5/5	

[a] NS, nonsignificant, $p > 0.5$.

TABLE VIII

Protection of BALB/c mice by adoptive transfer of spleen cells from mice immunized with 1 μg high m.w. IT-1 PS and 75 μg vinblastine (Vin)

Cells Given (raised to)	Amount ($\times 10^7$)	No. Survivors Total recipients	P Value vs Nonimmune Controls
1 μg PS + 75 μg Vin	0.25	0/5	NS[a]
	0.50	1/5	NS
	1.00	5/5	<0.001
	1.25	5/5	<0.001
1 μg PS only	0.50	0/5	NS
	1.00	0/5	NS
	10.00	0/5	NS
75 μg Vin only	0.50	0/5	NS
	1.00	0/5	NS
	10.00	0/5	NS
PBS (nonimmune)	0.50	0/5	
	1.00	1/5	
	10.00	0/5	

[a] NS, nonsignificant, $p > 0.5$.

date to destroy the immunogenic activity of IT-1 PS, and found that periodate treated IT-1 PS is not effective in eliciting immunity in conjunction with vinblastine. Finally, the inability of cyclophosphamide, a drug known to enhance T cell immune responses to protein antigens (11), to reproduce the effects of vinblastine further argues against a critical role for a protein contaminant.

The role of vinblastine in augmenting the establishment of PS-induced T cell-mediated immunity is unclear. Cyclophosphamide-sensitive T suppressor cells have been shown to be responsible for interfering with the development of delayed-type hypersensitivity reactions to sheep red blood cells (11, 16). The lack of protection elicited by cyclophosphamide plus PS suggests a different cell, other than cyclophosphamide-sensitive T suppressors, is affected by vinblastine. Kappler and Hoffman (17) have shown that vinblastine can block the production of antibody by interfering with the division of B cells. Also, when vinblastine is administered at the proper time, it does not block the division of T cells that give rise to helper cell functions (17). It is possible that by interfering with B cell division and augmenting T cell responses vinblastine allows BALB/c mice to develop immunity to the normally nonimmunogenic 1-μg dose of IT-1 PS. Another possibility is that the vinblastine affects a macrophage regulatory cell. Gorczynski (18) identified distinct macrophage subpopulations that are involved in regulating antibody and CMI responses, and this latter cell may be affected by the vinblastine. Because vinblastine exerts its effects by interfering with RNA synthesis (19), it could potentially affect nondividing cells like macrophages by interfering with protein synthesis needed to suppress the development of immunity. Another possibility is that the vinblastine acts by directly stimulating the effector T cells, which are reactive to the IT-1 PS antigen. Although this explanation seems unlikely, it is not excluded by the present study.

These data indicate that the traditional concepts about immunity to extracellular bacterial pathogens need to be broadened. We demonstrated the ability of T cells, in the absence of antibody, to mediate protective immunity in mice to live organism challenge with *P. aeruginosa* IT-1. Our production of an i.p. infection in mice should not be considered to be a model of natural infection caused by *P. aeruginosa* in the compromised host. The role of CMI in protection against *P. aeruginosa* in natural infections is unclear. Reynolds *et al.* (20) demonstrated the release of macrophage inhibition factor (MIF) from respiratory cell cultures of rabbits immunized with LPS from *P. aeruginosa* IT-2 when the cell cultures were challenged with nontoxic amounts of LPS. Antigen stimulated lymphocytes from spleens of immunized rabbits likewise produced MIF when cultured with nonimmune macrophages as indicator cells. However, this ability to produce MIF was transient, and, in another study, Reynolds (21) showed that in spite of MIF production, *in vitro* cultured alveolar macrophages from immune animals were not capable of enhanced phagocytosis and intracellular killing of *P. aeruginosa*. Harvath *et al.* (22), using a granulocytopenic dog as a model for *P. aeruginosa* infections, showed that animals actively immunized with LPS had better survival rates than unimmunized animals. However, passive transfer of immune sera did not protect recipient animals despite high levels of circulating antibody, suggesting that in the actively immunized dogs a cell-mediated immune mechanism was needed for full protection. Antibody-mediated opsonophagocytosis of *P. aeruginosa* is thought to be the major immune protective mechanism against *P. aeruginosa* infection (23). However, the existence of clinical circumstances in which *P. aeruginosa* infections persist, despite high levels of specific antibody, as seen in cystic fibrosis patients (24), dictates a need for further study of the importance of T cell-mediated immunity to *P. aeruginosa*.

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Cross-Protection by *Pseudomonas aeruginosa* Polysaccharides

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High-molecular-weight polysaccharide from *Pseudomonas aeruginosa* immunotypes 1 and 2 gave cross-protection in outbred CD-1 mice challenged with the heterologous immunotype organism. Both active immunization with 50 μg of polysaccharide, as well as passive transfer of immune serum were effective. The basis for this cross-protection is the ability of high doses of polysaccharide to induce antibody formation to both homologous and heterologous immunotype determinants.

Previous studies have shown that high-molecular-weight polysaccharide (PS)-type antigen can be isolated from cultural supernates or from the extracellular slime of *Pseudomonas aeruginosa* Fisher immunotypes 1 (IT-1) and 2 (IT-2) (11, 12). PS antigen appears to be a nontoxic, immunogenic form of the lipopolysaccharide (LPS) immunotype determinant. The PS antigens are composed principally of carbohydrates, with a molecular weight in the range of 1.5×10^5 to 2.5×10^5. They are nonpyrogenic in rabbits and nontoxic in mice (11, 12). PS antigen protects mice challenged with live organism 7 days after immunization with a single dose (10, 11).

It has also been shown that the PS antigens from IT-1 or IT-2 *P. aeruginosa* are cross-reactive with the respective O side chain PS of their lipopolysaccharide (LPS) (10–12). O side chains contain the immunotype-specific serological determinant for these strains of *P. aeruginosa* (1–3, 7, 8). To determine whether the protective immunity induced by PS antigens was based upon the immunotype-specific determinant shared between PS and LPS or whether the PS antigens showed cross-immunotype protection, we examined the ability of PS antigens from *P. aeruginosa* IT-1 and IT-2 to protect mice challenged with the live, heterologous organism. In addition, immune responses of rabbits and inbred mice to these two PS antigens was determined. Finally, the ability of intact LPS antigens to protect mice against homologous and heterologous strain challenge was also studied to compare the immunizing efficacy of PS and LPS antigens from *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* IT-1, IT-2, and IT-4 strains were used as previously described (5, 8, 10).

Antigens. High-molecular-weight PS and LPS were prepared as previously described (9, 11, 12).

Antisera. Rabbit antisera employed in serological assays and passive protective studies were prepared as previously described (11, 12). Murine antisera were obtained by retroorbital bleeding of ether-anesthetized mice 5 to 7 days after intraperitoneal immunization with a single dose of antigen in 0.5 ml of saline.

Adsorption of antisera. Antisera were adsorbed with the homologous or heterologous immunotype organism. Organisms used for adsorptions were grown in tryptic soy broth for 24 h, recovered by centrifugation, and killed by suspension in phosphate-buffered saline containing 1% Formalin for 24 h at room temperature. The organisms were washed three times with water and then lyophilized before their use as adsorbing antigens. Adsorptions with lyophilized cells (final concentration, 10 mg/ml of serum) were carried out at 4°C for 24 h with mixing.

Animal studies. Outbred CD-1 mice were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass. Inbred C3H/ANF and BALB/c mice were obtained from Cumberland View Farms, Clinton, Tenn. Animal studies for immunization-challenge experiments of active and passive protection were as previously described (10, 11). The challenge doses used here were chosen to routinely kill 90 to 100% of unimmunized mice.

Serological methods. The measurement of serum antibody levels to IT-1 and IT-2 were performed in a radioactive-antigen-binding assay as described by Farr (4). The antigen used in these assays was intrinsically labeled ^{14}C-PS, which was prepared from IT-1 and IT-2 organisms as follows. A 2-liter volume of Davis minimal media (Difco Laboratories, Detroit, Mich.) containing 1% sodium acetate instead of glucose as the carbon source was inoculated with growth from a tryptic soy agar culture of either the IT-1 or the IT-2 organisms. To this medium was added 20 mCi of [^{14}C]sodium acetate. The organisms were grown at 37°C with stirring for 48 h. The culture was centrifuged to remove the organisms and the supernates concentrated to a volume of 50 ml on an Amicon ultrafiltration TCF apparatus (Amicon Corp., Danvers, Mass.) utilizing PM 30 membranes. Crude PS antigen was precipitated from the concentrate by the addition of alcohol (80% vol/vol), and preparation of PS continued as previously described (9, 11). Specific activities for

TABLE 1. Ability of immunization with IT-1 and IT-2 PS antigens to afford protection in mice to challenge with live *P. aeruginosa* IT-1 and IT-2

Immunogen	Amt (μg)	Challenge		Survivors/total challenged[a]		
		Organism	No. ($\times 10^8$)	Expt 1	Expt 2	Expt 3
IT-1 PS	1	IT-1	3.2	0/10	1/10	1/10
	10			3/10	4/10	1/10
	50			9/10	10/10	10/10
	1	IT-2	3.8	0/10	0/10	1/10
	10			2/10	3/10	3/10
	50			5/10	5/10	6/10
	50	IT-4	1.2	1/10	0/10	1/10
IT-2 PS	1	IT-1	3.2	0/10	0/10	0/10
	10			6/10	4/10	3/10
	50			8/10	8/10	10/10
	1	IT-2	3.8	1/10	2/10	1/10
	10			7/10	7/10	5/10
	50			10/10	9/10	8/10
	50	IT-4	1.2	0/10	2/10	0/10
Saline		IT-1	3.2	0/10	0/10	1/10
		IT-2	3.8	1/10	0/10	0/10
		IT-4	1.2	0/10	0/10	1/10

[a] *P* value for protection of 50% or greater is ≤0.016 by Fisher exact test when there are no survivors in the control group; with one survivor in control group, *P* value for protection of 60% or greater is ≤0.027. Lower survival rates have *P* values > 0.05.

the PS antigens obtained were approximately 1.04 and 0.98 cpm/ng for the IT-1 PS and IT-2 PS, respectively.

Calculation of the concentration of specific antibody was as described (9).

RESULTS

Table 1 shows the results of three experiments examining the efficacy of cross-protection in outbred CD-1 mice actively immunized with the PS antigen from *P. aeruginosa* IT-1 and IT-2. Doses lower than 50 μg of IT-1 PS generally did not routinely protect a significant number of the outbred CD-1 mice from homologous IT-1 challenges. At a 50-μg dose, IT-1 PS was effective in eliciting 50 to 60% protection against challenge with the *P. aeruginosa* IT-2 cells. After a 50-μg dose of IT-2 PS, 80 to 100% protection against challenge with IT-1 cells was noted. IT-2 PS also had some cross protective capabilities at a 10-μg dose. Both IT-1 and IT-2 PS gave 90 to 100% protection after a 50-μg dose against challenge with homologous cells. Neither PS was capable of protecting against challenge with *P. aeruginosa* IT-4.

When LPS was used in cross-protection experiments, an LPS dose of 0.01 μg per mouse was effective in affording protection to the live homologous immunotype organism only (Table 2). Higher doses of LPS (10 to 100 μg) gave cross-protection similar to that seen with the PS antigens. These high doses of LPS were sublethal. Symptoms of endotoxin shock were noted in the animals at approximately 8 to 30 h postimmunization. These symptoms had completely subsided by the time of challenge 7 days later. The lack of protection to challenge with IT-4 organisms after immunization with these high doses of LPS indicates the specificity of this high-dose LPS cross-protection.

We next looked at the ability of rabbit antisera raised to the PS antigens from *P. aeruginosa* IT-1 and IT-2 to passively protect mice against live organism challenge. A 0.1-ml portion of rabbit antisera to the purified IT-1 and IT-2 PS antigens provided 100% protection to challenge with either the homologous or heterologous immunotype organisms (Table 3). Adsorption of antiserum raised to IT-1 PS with IT-1 cells removed the protective efficacy of this serum to *P. aeruginosa* IT-1 live cell challenge. However, protection against challenge with the heterologous strain of *P. aeruginosa* IT-2 remained.

When the antiserum raised to IT-1 PS was

adsorbed with IT-2 cells, passive protection against IT-2 *P. aeruginosa* was removed. However, there was no effect on passive protection against challenge with the homologous IT-1 strain. Similarly, adsorption of antiserum raised to the IT-2 PS with IT-2 cells eliminated passive protection to *P. aeruginosa* IT-2 live cell challenge. It did not significantly affect the protective efficacy against IT-1 live cell challenge. Antiserum raised to IT-2 PS adsorbed with IT-1 cells still conferred passive protection against IT-2 live cell challenge in mice, but had lost passive protection against *P. aeruginosa* IT-1 live cell challenge. When rabbit antisera raised to the LPS antigens from *P. aeruginosa* IT-1 and IT-2 were used in passive protection studies identical to those described above for antisera raised to the PS antigens, identical results were obtained (data not shown).

Serological studies. A radioactive-antigen-binding assay was employed to further define the nature of the cross-protective component of the PS antigens from *P. aeruginosa* IT-1 and IT-2. These studies used both hyperimmune rabbit antisera raised to the PS and LPS antigens and murine antisera raised to single doses of these antigens. Table 4 shows the concentration of specific antibody against the IT-1 and IT-2 PS in hyperimmune rabbit antisera raised to the IT-1 and IT-2 PS and LPS antigens. Antisera raised to PS and LPS from both strains contained antibody to both the IT-1 and IT-2 PS antigens. Adsorption of antisera raised to IT-1 PS and LPS antigens with the homologous IT-1 cells reduced the specific IT-1 PS antibody levels by 90% or more. This adsorption had little effect on the antibody level to IT-2 PS (Table 4). Similarly, adsorption of antisera raised to IT-2 PS and LPS with IT-2 cells reduced the specific IT-2 PS antibody below the level of detection in the radioactive-antigen-binding assay, but left intact appreciable amounts of antibody directed at the IT-1 PS (Table 4). IT-2 cells were able to remove some of the antibody to IT-1 PS from antisera raised to IT-2 PS and IT-2 LPS.

Further elucidation of the serological relatedness of IT-1 and IT-2 PS and LPS antigens was obtained by using mouse antisera raised to single immunizations of these antigens. It has been found (G. B. Pier and R. B. Markham, submitted for publication) that mice of the C3H/ANF strain are high responders to a single 1-μg dose of the IT-1 PS antigen, making both binding and protective antibodies. Mice of the BALB/c strain, on the other hand, make a minimal antibody response after a 25- to 50-μg dose of IT-1 PS. Data regarding the immune response of these two strains to high- and low-dose immunizations with the IT-1 and IT-2 PS and LPS antigens are shown in Table 5. After immunization with either 1 or 50 μg of the IT-1 PS, the C3H/ANF strain made primarily an immunotype-specific response. After immunization with 50 μg of IT-1 PS the BALB/c strain made almost equivalent antibody responses to the IT-1 and IT-2 PS antigens. Both mouse strains made a type-specific response to immunization with IT-1 LPS at a dosage of 0.10 μg, whereas at the high dose of 100 μg of IT-1 LPS, antibody to the IT-2 PS was elicited. After immunization with IT-2 PS both mouse strains produced antibody to IT-2 PS and antibody specific for IT-1 PS. Immunization with IT-2 LPS again produced an immunotype-specific response at low LPS doses and cross-immunotype antibody at high doses. Immunization with LPS generally gave a higher antibody level than did immunization with PS, except in C3H/ANF mice in which 50 μg of IT-1 PS induced a higher level of IT-1-specific antibody than did IT-1 LPS.

TABLE 2. Protection of mice after immunization with IT-1 and IT-2 LPS antigens and challenge with live *P. aeruginosa* IT-1 and IT-2

Immunogen	Amt (μg)	Challenge		Survivors/ total challenged[a]
		Organism	No. ($\times 10^8$)	
IT-1 LPS	0.01	IT-1	4.1	10/10
	0.01	IT-2	4.7	0/10
	0.10			0/10
	1.0			0/10
	10.0			3/10
	50.0			6/10
	100.0			7/10
	100.0	IT-4	1.1	0/10
IT-2 LPS	0.01	IT-2	4.7	10/10
	0.01	IT-1	4.1	0/10
	0.10			0/10
	1.0			0/10
	10.0			7/10
	50.0			9/10
	100.0			8/10
	100.0	IT-4	1.1	0/10
Saline		IT-1	4.1	0/10
		IT-2	4.7	1/10
		IT-4	1.1	1/10

[a] See Table 1, footnote *a* for *P* values.

DISCUSSION

These data indicate that the high-molecular-weight PS antigens obtained from *P. aeruginosa*

TABLE 3. Passive protection of mice given rabbit antisera raised to the PS antigens of *P. aeruginosa* IT-1 and IT-2

Antisera to:	Amt (ml)	Adsorbed with:	Challenge		Survivors/total challenged[a]
			Organism	No. ($\times 10^8$)	
IT-1 PS	0.1	—[b]	IT-1	1.6	10/10[a]
		—	IT-2	4.7	10/10
		—	IT-4	1.6	0/10
		IT-1 organisms	IT-1	1.6	0/10
		IT-1 organisms	IT-2	4.7	8/10
		IT-2 organisms	IT-1	1.6	10/10
		IT-2 organisms	IT-2	4.7	0/10
IT-2 PS	0.1	—	IT-1	1.6	9/10
		—	IT-2	4.7	10/10
		—	IT-4	1.6	0/10
		IT-1 organisms	IT-1	1.6	0/10
		IT-1 organisms	IT-2	4.7	10/10
		IT-2 organisms	IT-1	1.6	10/10
		IT-2 organisms	IT-2	4.7	0/10
NRS[c]	0.1	—	IT-1	1.6	0/10
		—	IT-2	4.7	0/10
		—	IT-4	1.6	0/10

[a] See Table 1, footnote *a* for *P* values.
[b] —, Not adsorbed.
[c] NRS, Normal rabbit serum.

IT-1 and IT-2 elicit cross-protective immunity when used as active immunogens in outbred mice. Antibodies raised to these PS antigens in rabbits are also cross-protective when passively transferred to mice. The basis for this cross-protection appears to reside in the generation of antibodies to the heterologous immunotype determinant that is present on the PS and shared with the O side chain portion of the LPS (11, 12). Antibody induced to the heterologous PS (heterotype antibody) could not be adsorbed out with cells from which the PS was isolated, whereas the antibody directed to the homologous PS was readily removed. Thus, high-molecular-weight PS and LPS antigens from *P. aeruginosa* IT-1 and IT-2 are cross-immunogenic at high doses, but do not appear to be antigenically cross-reactive. A similar cross-immunogenicity without cross-reactivity of capsular PS antigens from *Neisseria meningitidis* has been described (6). The generation of cross-protective antibodies by PS antigens suggests that a limited number of these antigens may be needed to produce a comprehensive multivalent vaccine to *P. aeruginosa*.

TABLE 4. Binding antibody in single pools of hyperimmune rabbit antisera raised to *P. aeruginosa* IT-1 and IT-2 PS antigens

Antisera raised to	Binding antibody (μg/ml)	
	IT-1 PS	IT-2 PS
IT-1 PS	43.9	10.7
Adsorbed with IT-1 organisms	<4[a]	10.6
Adsorbed with IT-2 organisms	30.2	<4
IT-1 LPS	105.7	82.6
Adsorbed with IT-1 organisms	9.7	82.4
Adsorbed with IT-2 organisms	108.9	<4
IT-2 PS	55.2	138.3
Adsorbed with IT-1 organisms	<4	143.2
Adsorbed with IT-2 organisms	32.3	<4
IT-2 LPS	80.2	214.2
Adsorbed with IT-1 organisms	4.5	166.8
Adsorbed with IT-2 organisms	28.8	<4

[a] Lower limit of detection of radioactive-antigen-binding assay for animal sera is 4.0 μg/ml.

The induction of heterotype antibodies after immunization with PS appears to be dependent on antigen dosage, the genetic constitution of the responding animal, and the particular PS antigen used. CD-1 mice require a high dose (50

TABLE 5. Binding antibody to the *P. aeruginosa* IT-1 and IT-2 PS antigens in mouse antisera after a single immunization with IT-1 and IT-2 PS and LPS

Mouse strain	Immunogen	Amt (μg)	Antibody (μg/ml) to[a]:	
			IT-1 PS	IT-2 PS
C3H/ANF	IT-1 PS	1	11.0 ± 0.8	<4
		50	63.0 ± 15.8	<4
	IT-1 LPS	0.1	38.4 ± 5.3	<4
		100.0	51.0 ± 6.2	8.7 ± 1.4
	IT-2 PS	1	5.2 ± 0.4	8.5 ± 1.1
		50	13.1 ± 1.3	29.2 ± 6.0
	IT-2 LPS	0.1	<4	48.2 ± 4.1
		100	13.3 ± 2.1	57.1 ± 8.3
BALB/c	IT-1 PS	1	<4	<4
		50	7.1 ± 1.3	6.5 ± 0.3
	IT-1 LPS	0.1	84.2 ± 4.3	<4
		100.0	68.5 ± 4.1	14.2 ± 3.8
	IT-2 PS	1	9.4 ± 1.9	12.1 ± 2.6
		50	16.2 ± 3.1	21.3 ± 5.8
	IT-2 LPS	0.1	<4	51.3 ± 9.4
		100	11.5 ± 2.3	46.9 ± 10.2

[a] Represents average concentration of antibody in five individual animal sera immunized with the indicated antigen dosage ± the standard error of the mean.

μg) of IT-1 and IT-2 PS for cross-protection. BALB/c mice and rabbits require a relatively high dose of IT-1 PS for antibody formation. C3H/ANF mice, however, required a low dose (1 μg) of IT-1 PS to induce specific antibody. Furthermore, the C3H/ANF mice responded to IT-1 PS immunization with only an IT-1 type-specific response, whereas BALB/c mice and hyperimmunized rabbits generated IT-2 PS-specific antibody after immunization with high doses of IT-1 PS. Thus, the dosage of PS and the genetic composition of the animals affected the threshold and specificity of the immune response. On the other hand, immunization with IT-2 PS produced both serotypic and heterotypic responses in all immunized animals, suggesting that this antigen may be a more broadly protective immunogen, and responses may be less restricted genetically. The antibody with specificity for IT-1 PS in rabbit hyperimmune serum that was raised to IT-2 PS may be more representative of the typical cross-reactive antibodies one would normally expect, since IT-2 organisms could adsorb out about 60% of the IT-1 PS antibody.

High doses of LPS were also found to elicit heterotype antibodies in mice. These doses of LPS were 10^1 to 10^4 times greater than the dose of LPS needed for type-specific antibody induction. The requirement for nearly equivalent amounts of LPS and PS for cross-protective immunity and antibody induction indicates that the clones of lymphocytes that recognize and respond to the heterologous immunotype determinant do so only at a certain dosage threshold. Furthermore, the chemical composition of PS (11, 12) shows only a low level (<1%) of potential LPS contamination of PS. Thus, for contaminating LPS to be responsible for the cross-immunogenicity of PS, the PS preparation would need to be 50 to 100% LPS, a possibility precluded by the chemical data. The ability of high doses of *P. aeruginosa* PS and LPS antigens to induce heterotype antibody may have a correlate in patients infected with *P. aeruginosa*. It has been shown (13) that in the convalescent serum from some patients infected with a single serotype of *P. aeruginosa*, antibody increases to heterologous serotypes of *P. aeruginosa* can be documented.

A possible explanation for cross-protection with IT-1 and IT-2 PS antigens is that these two serological determinants are closely related structural entities. PS antigens and the O PS side chain from LPS are serologically identical antigens (11, 12). PS antigens contain 5 to 7 individual monosaccharides, whereas O side chains from IT-1 and IT-2 LPS contain principally

rhamnose, glucose, and dideoxyhexosamines (11, 12). Since rhamnose, glucose, and dideoxyhexosamines are also part of PS antigens, the structural configuration of these monosaccharides most likely determines the epitope principally found on IT-1 and IT-2 PS. The differences in either the arrangement or linkages between glucose, rhamnose, and the dideoxyhexosamines must account for the serological distinction of the IT-1 and IT-2 strains of *P. aeruginosa*. After immunization with high doses of PS, antibodies to these closely related structures are induced, some with specificity for the PS inducing homologous antibody and some with specificity for the PS inducing heterologous antibody. The antibodies with specificity for the heterologous PS probably have a low affinity for the homologous PS antigenic determinant. This would explain the poor ability of organisms homologous to the inducing antigen to adsorb antibodies with specificity for the heterologous PS.

These experiments provide further insight into the nature of the serological determinants on *P. aeruginosa* antigens. It was shown that the specificity of mouse protection against live organism challenge with *P. aeruginosa* is dependent on the dosage of the immunizing antigen, the animal strain used to assess immunogenicity, and the PS antigen used for immunization. At low doses of LPS, only type-specific antibody responses and protection are seen. At high doses of PS and LPS, cross-protection and heterotype antibody responses occur in rabbits and some mouse strains. It required equivalent doses of intact LPS and PS to elicit heterotype protection in outbred CD-1 mice and cross-immunotype antibody in inbred BALB/c mice. Thus, low levels of contaminating LPS in the PS preparations cannot be held accountable for the efficacy of PS vaccination in mice.

PS antigens appear to be immunogenic forms of the *P. aeruginosa* LPS immunotype determinant. PS have previously been shown to differ from LPS O side chains, which bear the immunotype determinant (3, 7), by molecular size, monosaccharide constituents, and immunogenicity (10–12). Here it was shown that PS differs from intact LPS by its ability to elicit cross-protection in mice at doses equivalent to those required for homologous immunotype protection. Whether PS vaccines will have the same immunogenicity properties in humans as has been shown for mice will depend on the nature of the human immune response to this vaccine. PS from IT-1 *P. aeruginosa* has been shown to be immunogenic in healthy adult volunteers (9). The nature of the immunotype specificity of the human immune response elicited is currently being determined.

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Characterization of the Antibody Response in Inbred Mice to a High-Molecular-Weight Polysaccharide from *Pseudomonas aeruginosa* Immunotype 1

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We explored the genetic basis for the differing immune responses observed in inbred strains of mice to a high-molecular-weight polysaccharide (PS) from *Pseudomonas aeruginosa* immunotype 1 (IT-1). Previous studies have shown that C3H mice immunized with this antigen produce only immunotype-specific antibody. BALB/c mice immunized with IT-1 PS produce both anti-IT-1 PS antibody and antibody cross-reactive with PS from *P. aeruginosa* immunotype 2 (IT-2). In the current study, we observed that, in addition, these two strains differ in their ability to respond to low immunizing doses of IT-1 PS. C3H mice generated a protective antibody response after a 1-μg immunization, whereas BALB/c mice failed to produce protective antibody after receiving 1 μg of PS. Both strains generated protective levels of antibody after a 50-μg immunization. Genetic analysis of these response patterns indicates that the ability to produce cross-reactive antibody and the ability to respond to a 1-μg immunization are independently inherited traits. In addition, the responsiveness of C3H mice to a 1-μg immunization with the production of protective levels of antibody is not linked to the mouse major histocompatibility (H-2) complex, to sex-linked genes, or to a single gene outside the H-2 complex.

Pseudomonas aeruginosa organisms are extracellular gram-negative bacteria which cause significant morbidity and mortality in certain patient groups (6, 11–13). Because of the pathogenic potential of these bacteria and the difficulty in the antibiotic treatment of infections caused by them (12), efforts have been undertaken to develop a vaccine which would prevent this infection in patients at high risk (10).

One such candidate vaccine is a high-molecular-weight polysaccharide (PS) which has been isolated from the extracellular slime material of broth cultures from the Fisher-Devlin immunotype 1 (IT-1) strain of *P. aeruginosa* (10). Initial studies in outbred mice have demonstrated the protective potential of this vaccine against intraperitoneal (i.p.) challenge with live bacteria (9).

The ultimate success of any PS vaccine program will depend in part on the ability of individuals of heterogeneous genetic background to respond to the immunizing antigen. Previous findings in inbred mice (8) have indicated that after immunization with IT-1 PS, BALB/c and C3H mice differ in their ability to produce antibody cross-reactive with Fisher-Devlin *P. aeruginosa* immunotype 2 (IT-2) PS. In the current report, we document an additional difference in the response pattern of these two inbred strains. C3H mice produce antibody after immunization with low doses (1 μg) of antigen. BALB/c mice do not. We studied whether this difference is genetically linked to the ability to produce cross-reactive antibody, to the mouse major histocompatibility (H-2) complex, or to a single gene outside the H-2 complex. Our studies indicate that the two traits that distinguish C3H and BALB/c responses are inherited independently and that the ability to respond to a limiting immunizing dose is not linked to sex, the H-2 complex, or a single gene outside the H-2 complex.

MATERIALS AND METHODS

Mice. Female inbred BALB/c mice, C3H/ANF mice, F_1 mice, and back-crosses of these inbred strains, 8 weeks of age, were obtained from Cumberland View Farms, Clinton, Tenn. C3H.SW, C3H/HeJ, CBA/J, C57B1/6J, AKR/J, and C57Br/cdJ mice were obtained from Jackson Laboratories, Bar Harbor, Maine.

Detection of antibody-producing cells. Direct and indirect plaque-forming cells (PFC) specific for *P.*

aeruginosa IT-1 PS were detected by a slide version of the technique of localized hemolysis-in-gel described by Jerne et al. (4). To detect IT-1 PS-specific PFC, sheep erythrocytes (SRBC) were sensitized by mixing 0.5 ml of washed packed SRBC, 1 ml of a 1 mg/ml solution of PS in 0.15 M NaCl, and a 0.07% chromium chloride solution. The mixture was allowed to stand for 5 min, and the sensitized cells were then washed three times in normal saline and used in a final concentration of 10% (vol/vol). Background PFC to unsensitized SRBC were determined for each result and subtracted from the total PFC detected by using sensitized SRBC.

Antisera. Heavy chain-specific goat anti-mouse immunoglobulin M (IgM) was included in the agarose to inhibit IgM PFC when non-IgM PFC were detected. Heavy and light chain-specific rabbit anti-mouse IgG was used for the detection of non-IgM PFC. Both antisera were obtained from Cappel Laboratories, Cochranville, Pa.

Antigen and immunization. PS from Fisher-Devlin IT-1 *P. aeruginosa* was isolated and purified by previously described techniques (7, 10). Briefly, 95% alcohol-precipitable material from the supernatant of a tryptic soy broth (Difco Laboratories, Detroit, Mich.) culture of *P. aeruginosa* was collected, dissolved in 1% acetic acid, adjusted to pH 5.0 with glacial acetic acid, and heated at 90°C for 18 h. The supernatant was then collected, extracted with chloroform and phenol, and then precipitated with 95% alcohol. The precipitate was then dissolved in phosphate-buffered saline and applied to a Sephacyl S-300 column (2.6 by 100 cm). The serologically active material eluting between the void volume and the point at which a 70,000-molecular-weight dextran marker begins to elute was collected, precipitated with alcohol, dialyzed, and lyophilized. The lyophilized material was reconstituted in phospate-buffered saline before injection. Mice were immunized by i.p. injection of the PS in the specified dose dissolved in 0.5 ml of 0.15 M NaCl.

Challenge with live organisms. Organisms for challenge of immunized and control mice were grown for approximately 16 h on tryptic soy agar. One week after immunization, mice were challenged by i.p. inoculation with a dose of *P. aeruginosa* IT-1 (1.3×10^8 organisms) that routinely kills 90 to 100% of nonimmune mice. The mean lethal dose for this organism in these mice by the i.p. challenge route is 5×10^7 organisms (G. Pier, unpublished data).

Radioactive antigen-binding assay. ^{14}C intrinsically labeled PS from *P. aeruginosa* IT-1 and IT-2 were prepared from 2 liters of Davis minimal media (Difco) containing 20 mCi of ^{14}C sodium acetate and 1% cold sodium acetate. Specific activities of 1.0 and 0.98 cpm/ng for the IT-1 PS and IT-2 PS were obtained, respectively. A radioactive antigen-binding assay was performed by the method of Farr (3), and quantification of the amount of antibody (micrograms per milliliter) determined as previously described (7).

Statistics. Student's *t* test (1) was used to evaluate the significance of the differences observed in the generation of antibody or antibody-producing cells. The significance of differences in survival between inbred mouse strains was determined by a Fisher exact test (1).

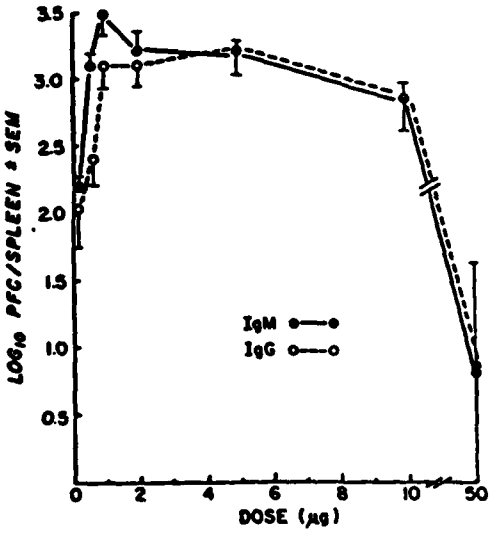

FIG. 1. Log_{10} IgM and IgG PFC per spleen ± standard error of the mean versus the immunizing dose of IT-1 PS in C3H/ANF mice, determined 5 days after immunization.

RESULTS

Dose response relationships in inbred mice. Groups of five C3H/ANF mice were immunized with different doses of IT-1 PS ranging from 1.0 to 50 μg. Five days after immunization, the number of spleen cells producing either IgG or IgM antibody specific for IT-1 PS was determined. Previous kinetic studies had demonstrated that the optimal PFC response occurred on day 5 after immunization. As seen in Fig. 1, the maximum number (2,800) of IgM-secreting cells was generated with an immunizing dose of 1 μg. The optimal IgG response occurred after a 5-μg immunization, but this response did not differ significantly from that seen at 1 μg ($P > 0.5$). At 50 μg, few if any IgG or IgM PFC could be detected. With the same protocol, BALB/c mice failed to produce any detectable IgM or IgG PFC after immunizing doses of IT-1 PS ranging from 1.0 to 50 μg. Kinetics of the PFC response for BALB/c mice were determined by using immunizing doses of 1 and 50 μg. No splenic PFC could be detected on days 3 through 10 after immunization.

Despite the absence of PFC in the spleens of BALB/c mice, antibody could be detected in the sera of those BALB/c mice immunized with 50 μg of IT-1 PS by use of the radioactive antigen-binding assay (Table 1). As in the plaque assay, however, immunization with 1 μg of antigen failed to produce a significant rise in antibody over preimmunization levels. By contrast,

TABLE 1. Ability of C3H/ANF and BALB/c mice to produce antibody after immunization with 1 and 50 μg of IT-1 PS

Mouse strain	Immunizing dose (μg)	Serum antibody concentrations (μg/ml)
C3H/ANF	1	17.6[a]
BALB/c	1	<4.0[b]
C3H/ANF	50	28.1
BALB/c	50	7.1

[a] Number represents mean antibody concentration detected in the sera of five immunized mice.

[b] The lower limit for the detection of antibody is 4 μg/ml.

C3H/ANF mice did produce significant amounts of serum antibody after both a 1- and a 50-μg immunization, despite the absence of detectable splenic PFC after the 50-μg dose. These relatively high antibody levels in C3H mice given 50 μg of PS suggest either that antibody was being produced at a site other than the spleen or, less likely, that an isotype was being produced after high-dose immunization that could not be detected in the plaque assay.

Relationship between immunizing dose and ability to withstand challenge with *P. aeruginosa* IT-1. To examine the protective efficacy of PS immunization in inbred mice, groups of five C3H/ANF and BALB/c mice were immunized once with IT-1 PS in doses ranging from 1.0 to 50 μg. One week later, the mice were challenged with 1.3×10^8 *P. aeruginosa* IT-1 organisms. Among BALB/c mice, the only group with significant survival was that which had received the 50-μg immunizing dose (Table 2). No BALB/c mice in the other immunization groups survived this challenge.

C3H/ANF mice challenged with an equivalent number of *P. aeruginosa* IT-1 organisms also showed protection provided by the 50-μg immunizing dose. However, the ability of this mouse strain to respond to lower doses of PS was again confirmed by the protective efficacy of the 1- and 5-μg immunization doses, with 100% survival seen in all immunized groups.

Ability of (BALB/c × C3H)F_1 mice to produce anti-IT-2 PS antibodies after immunization with IT-1 PS. Previous experiments have indicated that C3H/ANF mice produce only an anti-IT 1 PS type-specific response after immunization with 50 μg of IT-1 PS. After the same immunization, BALB/c mice produce both anti-IT-1 PS and anti-IT-2 PS antibodies (8). We considered the possibility that C3H mice processed the PS in a manner that both permitted them to respond to lower immunizing doses (1 μg) and precluded their producing the anti-IT-2 PS cross-reactive antibody.

To evaluate this hypothesis, we examined the response patterns of (BALB/c × C3H)F_1 mice. Pooled sera from (BALB/c × C3H/ANF)F_1 obtained before immunization contained less than 4 μg of antibody per ml against IT-1 PS. Six days after immunization with 1 μg of polysaccharide, pooled sera from these mice contained 41 μg of antibody to IT-1 PS per ml. No increase in antibody titers to IT-2 PS above the preimmune level (4 μg/ml) was observed after immunization with 1 μg of IT-1 PS. However, after immunization with 50 μg of IT-1 PS, the level of anti-IT-2 PS antibody increased to 11.2 μg/ml over a preimmune level of less than 4 μg/ml. The antibody titer to IT-1 PS increased to 32.6 μg/ml (from less than 4 μg/ml) in this same group of mice. These results indicate that processing the antigen in a manner that permits an antibody response to low immunizing doses does not preclude production of the cross-reactive antibody in response to high-dose immunization. Therefore, the inability of the parental C3H mice to produce cross-reactive antibody is not linked to their ability to respond to a 1-μg immunization.

Genetics of response to 1 μg of IT-1 PS. Since C3H/ANF ($H\text{-}2^k$) and BALB/c ($H\text{-}2^d$) mice differ at the H-2 complex, we next studied whether the ability to respond to the 1-μg dose of IT-1 PS was H-2 linked. Groups of five mice of different H-2 types and different backgrounds were immunized with 1 μg of IT-1 PS, including strains C3H/HeJ, C3H/HeDiSn, C57Br/cdJ, CBA/N, C58/J, CBA/J (all $H\text{-}2^k$), C57B1/6J ($H\text{-}2^b$), and C3H.SW.Sn ($H\text{-}2^b$ on C3H background). Five days later, the generation of IgM splenic PFC was determined. Only strains of mice with a C3H background generated splenic PFC after immunization with 1 μg of IT-1 PS. Mice of another $H\text{-}2^k$ strain, CBA/J, failed to respond, and strains of C3H background, including the C3H.SW strain ($H\text{-}2^b$), did respond. PFC responses in all responding strains were equivalent

TABLE 2. Survival of BALB/c and C3H mice challenged with live *P. aeruginosa* IT-1 after immunization with different doses of IT-1 PS

Immunizing dose (μg)[a]	No. of BALB/c mice surviving/no. challenged	*P* value[b]	No. of C3H mice surviving/no. challenged	*P* value[b]
0	0.5		0/5	
1	0/5	NS[c]	5/5	0.01
5	0.5	NS	5/5	0.01
50	4/5	0.05	5/5	0.01

[a] Quantity of IT-1 PS given i.p. 7 days before challenge.

[b] Significance of difference in number of survivors compared to mice of the same strain receiving no prior immunization, determined by the Fisher exact test.

[c] NS, Not significant.

in the range of 3,000 to 4,000 PFC per spleen. It is apparent that the ability to respond is determined by genes that do not fall within the H-2 complex. CBA/J and C3H/HeJ mice have the same IgG1 allotype, and the failure of the CBA/J mice to respond to a 1-μg dose indicates that responsiveness is also not linked to the IgG2a allotype locus. Testing of the sera from these mice in the radioactive antigen-binding assay confirmed the lack of response to a 1-μg dose of IT-1 PS (data not shown).

To determine whether the ability to respond to a 1-μg immunization is sex linked, we examined the response patterns in F_1 male offspring of both (BALB/c × C3H)F_1 and (C3H × BALB/c)F_1 origin. The responses of both of these reciprocal F_1 mice were equivalent ($P > 0.05$; Table 3), indicating that the response to 1 μg of PS is not sex linked.

To evaluate whether the response to 1 μg is linked to a single gene outside the H-2 complex, we studied the response pattern in backcrosses of F_1 mice with both of the parental strains. If responsiveness were linked to a single dominant gene, 100% of the offspring of C3H × F_1 backcrosses would respond to a 1-μg immunization, and 50% of the offspring of BALB/c × F_1 backcrosses would respond. In fact, 95% of C3H/ANF × F_1 mice did respond, but 70% of BALB/c × F_1 mice also responded by a twofold or greater (>8 μg/ml) increase in serum antibody levels. The response patterns therefore do not segregate into a pattern consistent with linkage of responsiveness to a single gene.

DISCUSSION

In the current investigation, we characterized antibody responses of inbred mouse strains to a PS antigen isolated from one immunotype of the gram-negative bacterium *P. aeruginosa*. Our findings indicate that inbred mice can be used to identify two distinct response patterns which vary both by the limiting dose of antigen which can stimulate a protective antibody response and by the specificity of the antibodies which are generated.

TABLE 3. Serum antibody levels and splenic PFC produced by (C3H × BALB/c)F_1 and (BALB/c × C3H)F_1 male mice after immunization with 1 μg of IT-1 PS[a]

Strain	Antibody level (μg/ml)[b]	PFC[c]
(C3H × BALB/c)F_1	11.9 ± 4.6	5118 ± 2964
(BALB/c × C3H)F_1	7.5 ± 1.2	6330 ± 2077

[a] As determined by the Student *t*-test, the responses of both of these reciprocal F_1 hybrids in antibody and PFC production were equivalent: $P > 0.05$.

[b] Mean antibody ± standard deviation determined in sera of five mice 5 days after immunization.

[c] Mean ± standard deviation of number of PFC per spleen directed at IT-1 PS-coated SRBC 5 days after immunization.

All substrains of C3H mice we evaluated were capable of producing antibody after immunization with 1 μg of PS, and antibody production was associated with the appearance of PFC in the spleen. At higher immunizing doses (50 μg), splenic PFC disappeared, although antibody did appear in the serum and was apparently being produced at other sites. This decline in splenic PFC with higher immunizing doses has been described for other PS antigens (5).

Antibody-producing cells which are activated by low doses of PS do not appear in the spleens of BALB/c mice, and this strain produces no serum antibody after a 1-μg immunization. Like C3H mice, BALB/c mice do produce serum antibody in nonsplenic lymphoid organs after a 50-μg immunization, but, as indicated in previous studies, this antibody is of broader specificity than that produced by C3H mice (8).

Because BALB/c mice produced antibody that cross-reacts with IT-2 PS and C3H mice do not, the most logical explanation for the different responses of these two strains would be that they are recognizing different determinants on the immunizing IT-1 PS antigen. By this model, the determinant recognized by the BALB/c mice would be structurally similar to determinants found on IT-2 PS, and BALB/c mice would therefore produce a cross-reactive antibody after immunization with IT-1 PS. However, previous studies have shown that the anti-IT-2 PS antibody produced by BALB/c mice cannot be absorbed with IT-1 organisms or PS (8). It appears then that BALB/c mice process this antigen in a manner that stimulates a heteroclitic antibody response to IT-2 PS; i.e., the anti-IT-2 PS antibody produced reacts with this heterologous antigen but fails to react with the homologous immunizing antigen, IT-1 PS.

The fact that C3H mice generate splenic PFC and BALB/c mice do not suggests further that the IT-1 PS antigen is processed differently in these two strains. We sought to determine, therefore, whether the two characteristics that distinguish the response of these two mouse strains were linked: does the ability to respond to a 1-μg immunization and to generate splenic PFC preclude the ability to produce the heteroclitic anti-IT-2 PS response after immunization with 50 μg of PS?

The data for the (BALB/c × C3H)F_1 mice indicate that these two traits are not linked. The F_1 mice are capable both of generating antibody and splenic PFC after a 1-μg immunization and of producing the cross-reactive antibody after a 50-μg immunization. We have found, then, that

these two inbred strains differ in their response to IT-1 PS in two important respects: (i) the optimal immunizing dose of IT-1 PS is different, and (ii) the potential ability to induce cross-immunotype protection with a single vaccine type is also different.

Studies with ovalbumin by Dunham and coworkers (2) provide precedent for the observation that limiting the immunizing dose of antigen restricts the number of inbred mouse strains capable of responding. In those experiments, most strains tested produced antibody after immunization with high doses of antigen, but only strains of a few H-2 types could respond to a limiting dose of antigen. Dunham et al. suggested that at a sufficiently small dose, only a single determinant of the complex ovalbumin molecule is present in enough quantity to elicit an antibody response and that H-2-linked genes determine the ability of different inbred strains to respond to that determinant.

Although the ability of C3H mice to respond to the low dose of PS appears superficially to resemble this ovalbumin model, our studies indicate that the genetic basis for the two responses is quite different. Specifically, the ability to respond to a low does of IT-1 PS is not H-2 linked. C3H.SW mice (H-2^b) have a genetic background which is identical to that of other C3H mice but different from that of the other C3H mice tested (H-2^k) at the major histocompatibility complex. The C3H.SW mice are still able to generate a typical C3H response to 1 μg of IT-1 PS. It therefore appears that the characteristics which allow the response to 1 μg of IT-1 PS reside in genes outside the H-2 complex.

In addition, the cross-breeding studies of F_1 offspring by the responder or nonresponder parental strain indicate that the ability to respond to a 1-μg immunizing dose of IT-1 PS is not determined by a single gene outside the H-2 complex. In both parental crosses, a broad range of antibody responses develops (data not shown). There does seem to be a gene-dose effect, however, in that the parental by F_1 backcross that would be expected to have the greatest number of C3H genes (F_1 × C3H) has a greater number of responders than the mice with a larger proportion of BALB/c genes (F_1 × BALB/c).

If the patterns of responsiveness observed in these inbred mice are maintained in humans, they would have important implications for a vaccine program. First, although some individuals might be able to respond to a low vaccine dose, higher doses will be required to generate adequate responses in the greatest number of individuals. Second, although after immunization with IT-1 PS some individuals might produce antibodies cross-reactive with IT-2 PS, separate immunization with IT-2 PS will be required to ensure that all individuals have antibody to that immunotype. Human studies which have been reported (9) or are in progress (G. Pier and D. Thomas, submitted for publication) indicate that the patterns of responsiveness observed in these inbred mice are in fact preserved in human responses to this PS antigen.

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Characterization of the Human Immune Response to a Polysaccharide Vaccine from *Pseudomonas aeruginosa*

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Sera from humans vaccinated with a high-molecular-weight polysaccharide vaccine to *Pseudomonas aeruginosa* immunotype 1 (IT-1) were analyzed for duration of the immune response, specificity for the IT-1 determinant, and by assessing the immunoglobulin classes elicited. The ability of purified IgG, IgM, and IgA to interact with peripheral blood leukocytes, as well as purified polymorphonuclear neutrophils or mononuclear cells, was also examined in an opsonophagocytosis assay. Levels of antibody to IT-1 remained significantly ($P < 0.001$) elevated 21 months after immunization. Responses were generally specific to the IT-1 serotype determinant. Some vaccinees also responded to immunotype 2 and immunotype 5 determinants. IgG, IgM, and IgA serum antibodies were all elicited by vaccination. IgG and IgA were effective opsonins for *P aeruginosa*. IgM-mediated opsonophagocytosis required complement. Serum IgA was highly effective in conjunction with mononuclear cells in opsonophagocytosis of *P aeruginosa*, suggesting that these immune components may be capable of protecting neutropenic hosts.

The role of antibody, complement, and phagocytic cells in human immunity to infection with *Pseudomonas aeruginosa* is well documented. Studies in animals [1] and patients [2–4] have suggested that antibodies to the lipopolysaccharide are associated with protection from death related to *P aeruginosa* sepsis. The requirement for phagocytic cells is indicated by the increased susceptibility of leukopenic patients to *P aeruginosa* infection [5] and by the resistance to bactericidal effects of antibody and complement that is shown by most strains of *P aeruginosa* [6, 7]. One study [4] has demonstrated a protective effect of antibody to the lipopolysaccharide and exotoxin A that is independent of the PMN level – an observation suggesting that antibody-mediated resistance may be independent of white blood cells. Thus, it appears possible that the chances of surviving *P aeruginosa* sepsis may be increased in persons whose levels of type-specific antibody can be augmented by vaccination.

Vaccines against *P aeruginosa* that have been tested in the past have contained lipopolysaccharide [8–10]. The toxicity of these preparations has precluded their routine use in patients. We have been exploring the vaccine potential of a high-molecular-weight polysaccharide preparation isolated from culture supernatants of *P aeruginosa*. High-molecular-weight polysaccharides contain the serotype (immunotype) determinant that is also found on the "O"-polysaccharide side chain of the lipopolysaccharide. However, high-molecular-weight polysaccharides differ from the O-polysaccharide side chain by molecular size, monosaccharide composition, and immunogenicity in animals [11–13]. In addition, high-molecular-weight polysaccharides differ from the lipopolysaccharide in terms of toxicity, monosaccharide composition, and the absence of lipids [11–13]. Our previous report [14] indicated that the high-molecular-

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weight polysaccharide vaccine prepared from an immunotype 1 (IT-1) strain of *P aeruginosa* was immunogenic in adult volunteers, inducing significant increases in both binding and opsonic antibody by two weeks after immunization. The only adverse reaction to the vaccine was a slightly sore and tender arm at the site of injection 24 hr after vaccination. In this report we characterize further the immune response to this *P aeruginosa* vaccine.

Materials and Methods

Antisera. Antisera were obtained from volunteers immunized with one lot of a high-molecular-weight polysaccharide vaccine prepared from IT-1 *P aeruginosa* [14]. The collected sera were stored at −20 C.

Serologic methods. Levels of antibody were determined by means of a quantitative radioactive antigen-binding assay [14, 15]. The antigens used in this assay were intrinsically ^{14}C-labeled polysaccharide antigens prepared from the seven Fisher immunotype strains of *P aeruginosa* [16]. Each polysaccharide shared serologic specificity with the O-polysaccharide side chain on the corresponding lipopolysaccharide [17].

The classes of immunoglobulin present in serum obtained before and after immunization were determined in a radioimmunoprecipitin test [18]. Each antiserum was allowed to react for 2 hr at 37 C with five times the amount of ^{14}C-labeled polysaccharide known to bind completely to the antibody present in that antiserum. After this period, antiserum to human IgG, IgM, or IgA (Miles Biochemicals, Elkhart, Ind) was added to the test mixtures. The amount added was determined for each lot of antiserum in a quantitative precipitin assay in which 100–200 μl of antiserum to immunoglobulin/100 μl of test serum was generally used. The antiserum to immunoglobulin was allowed to precipitate the immunoglobulin present in the test sera during incubation at 4 C for 18 hr. Precipitates were centrifuged and washed once with 0.5 ml of ice-cold PBS. The pellets were dissolved in Protosol® and Liquiflor/Toluene® (New England Nuclear, Boston, Mass) and counted in a scintillation counter. The percentage of each immunoglobulin isotype present in the test sera was calculated by the following formula: (cpm bound by individual immunoglobulin isotype − background) × 100/(cpm bound by all three isotypes − background).

Opsonophagocytic assay. Opsonophagocytosis of live IT-1 *P aeruginosa* was performed as previously described [14]. In some experiments the organisms were opsonized with specific immunoglobulin isotypes prior to the addition of phagocytic cells and complement. Immunoglobulins and bacteria were incubated for 30 min at 4 C, and the bacteria then were washed with minimal essential medium (MEM). The opsonized and washed bacteria were added to a suspension of human peripheral-blood leukocytes and a 1:5 dilution of guinea pig complement for determination of killing during incubation at 37 C. The percentage killed at each time point was determined by the following formula: percentage killed = 100 − ([number of bacteria alive × 100]/[number of bacteria inoculated into tube]).

Purification of phagocytic cells. Peripheral-blood leukocytes were obtained by sedimentation of whole venous blood on a dextran T-70 gradient, as described [14]. Leukocytes were separated into monocyte and PMN populations by density-gradient centrifugation of heparinized whole venous blood on lymphocyte separation medium (Litton Bionetics, Kensington, Md). The mononuclear cell band was recovered, washed three times with MEM, and resuspended to the desired concentration (10^7 live cells/ml) in MEM after counting with trypan blue dye. The PMN pellet was resuspended in MEM and placed on a dextran T-70 gradient for further purification, as described [14]. These cells were also suspended in MEM to a concentration of 10^7 live cells/ml after isolation. The purity of the cell populations was checked by differential counting after staining with Wright's stain. Purity of >95% was always observed.

Separation of immunoglobulin isotypes. Immunoglobulin isotypes were separated into purified IgG, IgM, and IgA by two methods. The first was the in-line chromatographic separation of immunoglobulin isotypes described by Griffiss et al [19]. In the second procedure, the removal of IgM by binding to QAE Sephadex (IgMsorb® columns; Isolab, Akron, Ohio) was followed by the recovery of the eluate containing IgG and IgA. The IgM was eluted from the Sephadex column as recommended by the manufacturer and was exchanged into MEM by passage over a PD-10 salt exchanger column (Pharmacia Fine Chemicals, Piscataway, NJ). The IgM was stored at −80 C. The eluate containing IgG and IgA was next passed over a column of protein A Sepharose (Pharma-

cia) for binding of the IgG. The IgA fraction was then recovered as the eluate from the Sepharose column. The IgG that was bound to the column was eluted with 0.1 *M* glycine-HCl buffer (pH 3.0), exchanged into MEM over a PD-10 column, and stored at − 80 C. The IgA-containing eluate of the protein A-Sepharose column was passed over a column of antiserum to human IgG and antiserum to human IgM covalently linked to Sepharose 4B. The eluate from this column was collected, exchanged into MEM, and stored at − 80 C. The immunoglobulin isotype level in each of the fractions collected was measured on Tri-Partigen® single radioimmunodiffusion test plates (Calbiochem-Behring, La Jolla, Calif). The purity of these immunoglobulin isotype preparations was checked; LC-Partigen® plates (Calbiochem-Behring) were used for detection of low levels of specific immunoglobulin. Only preparations showing >97% purity of the desired isotype were used in the experiments that have been described.

Statistics. Statistical differences in levels of antibody were calculated by a *t*-test.

Results

Duration of antibody response. Of the 42 persons who received the polysaccharide vaccine, 32 responded with a twofold or greater increase in levels of antibody binding [14]. Table 1 documents the geometric mean levels of specific antibody six to nine months and 16-21 months after immunization in some of these 32 vaccinees. Our previously published data on antibody levels at six months included only 12 vaccinees who received a single dose (150 μg) of vaccine [14]. Six to nine months and 16-21 months after vaccination, specific antibody levels remained significantly ($P < 0.001$) elevated over values before immunization. The antibody levels observed at 16-21 months appear to be higher than those at six to nine months, but the group available for antibody determinations at the latter time was only a small subset of the initial group of vaccinees. The results indicate that the single dose of vaccine was sufficient for the maintenance of elevated levels of specific antibody over an extended period.

Response to heterologous P aeruginosa polysaccharide immunotypes. Each sample of immune serum collected after immunization with the IT-1 polysaccharide was examined for increased levels of antibody to the high-molecular-weight polysaccharide isolated from *P aeruginosa* of immunotypes 2-7 (IT-2 through IT-7), as described by Fisher et al [16]. Of the 42 persons immunized (seven with 50 μg, five with 75 μg, 12 with 150 μg, and 18 with 250 μg), seven had elevated levels (twofold or greater increase) of antibody to the IT-2 polysaccharide two to four weeks after immunization, and three had elevated levels to the

Table 1. Duration of immune response in recipients of polysaccharide vaccine against IT-1 *P aeruginosa*.

Time (no. of vaccinees)	Geometric mean (± SD) antibody level (μg/ml)
Before vaccination (30)	6.4 ± 2.0
After vaccination	
6-9 months (30)	23.0 ± 3.3*
16-21 months (11)†	47.9 ± 2.9*

* $P < 0.001$

† Eleven of the 30 vaccinees available for antibody determination six months after immunization were also available at 16-21 months

Table 2. Immunoglobulin isotypes in serum from recipients of *P aeruginosa* IT-1 polysaccharide vaccine.

Dose in μg, time, (no. of vaccinees)	Total antigen bound by serum (%)†		
	IgG	IgM	IgA
250			
Before immunization (12)	54.7	16.6	28.6
After immunization			
28 days (13)	48.6	11.6	39.7
6 months (10)	48.7	12.0	39.3
150			
Before immunization (11)	49.1	29.6	21.3
After immunization			
28 days (8)	56.5	28.0	15.5
6 months (10)	55.4	28.3	16.3
75			
Before immunization (2)	59.8	12.2	18.1
28 days after immunization (2)	37.1	11.4	51.4
50			
Before immunization (3)	53.7	5.8	37.8
28 days after immunization (3)	58.3	16.6	23.8

* The number of vaccinees varies because in some instances the level of antibody binding was insufficient for the detection of immunoglobulin isotypes and in some instances sera were unavailable for study. Six-month serum samples from recipients of 50- and 75-μg doses were not studied because an insufficient number were available.

† Results represent average percentages of specific immunoglobulin isotypes found in all sera studied

IT-5 polysaccharide at this time. Six of the seven persons responding to the IT-2 polysaccharide and all three responding to the IT-5 polysaccharide received the largest dose of vaccine. Thus, all but one of the persons receiving smaller doses responded only to the IT-1 polysaccharide. These data are consistent with previously reported results in animals; in those studies, responses to heterologous polysaccharide determinants occurred only after large doses of the polysaccharide vaccine [20].

Immunoglobulin isotypes associated with response to the IT-1 polysaccharide vaccine. Table 2 shows the percentages of IT-1 polysaccharide-specific IgG, IgM, and IgA in sera of vaccinees. These determinations were made in specimens of serum obtained before immunization, 28 days after immunization, and six to nine months after immunization. The relative proportions of IT-1 polysaccharide-specific IgG, IgM, and IgA remained unchanged after immunization, although binding levels were considerably higher on day 28 and later than they had been before immunization (table 1). IgM was seen in a few vaccinees, in some cases for as long as six to nine months after immunization. IgG was the predominant antibody in most vaccinees, but IgA also accounted for a significant proportion of the IT-1 polysaccharide-specific antibody response.

Opsonophagocytosis of IT-1 P aeruginosa with isolated immunoglobulin isotypes. Purified immunoglobulin isotypes (purity, >97%) were obtained from selected immune sera and tested for their ability to mediate opsonophagocytosis by peripheral-blood leukocytes. Figure 1 shows the ability of whole serum and isolated IgG, IgM, and IgA—with and without added complement—to mediate phagocytic killing of *P aeruginosa* during 1 hr. In this assay all of the components (immunoglobulin isotype or serum, phagocytic cells, complement, and bacteria) were added together at the start of the test. The amount of immunoglobulin was always greater than that needed for maximal binding to bacterial cells. Optimal killing at 60 min was obtained with IgG plus complement; the level of killing was analogous to that with whole serum plus complement. Deletion of complement from the reaction mixture decreased IgG-mediated killing by ~0.5 log (20% fewer bacteria killed).

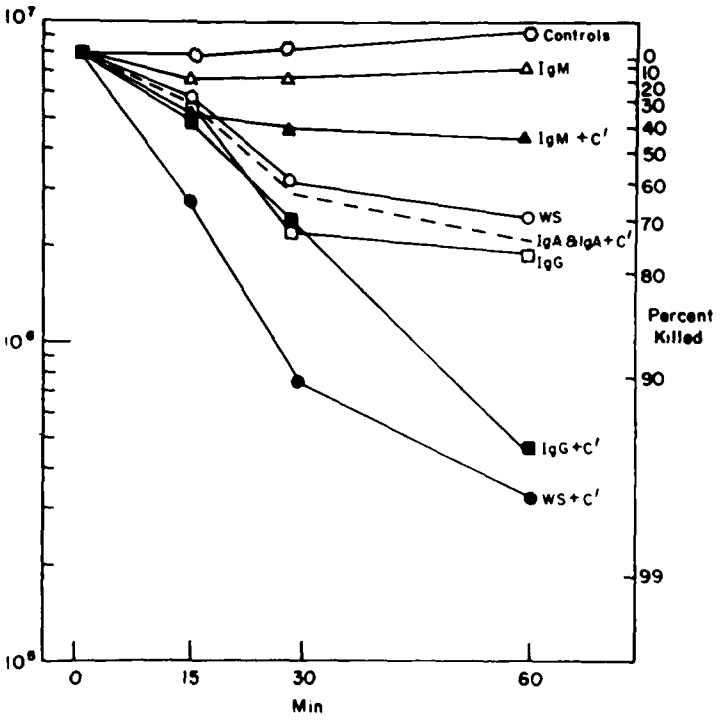

Figure 1. Opsonophagocytic killing of IT-1 *P aeruginosa* by whole serum (WS) and isolated immunoglobulin isotypes, with and without complement (C'), over a 60-min period of incubation at 37 C. Left ordinate shows counts of bacteria surviving; right ordinate shows percentages of input inoculum killed.

This result was comparable to the decrease in killing with whole serum in the absence of complement (0.8 log, or 25% fewer bacteria killed).

IgA antibody was capable of mediating killing of ~0.55 log (75%) of the input inoculum at 1 hr. The addition of complement had no effect on IgA-mediated opsonic killing. IgM plus complement killed ~0.3 log (45%) of the input inoculum by 1 hr. Deletion of complement from the IgM reaction mixture essentially removed the ability of IgM to mediate opsonic killing of *P aeruginosa*.

Next, the ability of serum IgA to inhibit or block IgG- and IgM-mediated opsonophagocytosis was tested. Organisms were first coated with IgA for 30 min at 4 C and washed; immune IgG or IgM plus complement and peripheral blood leukocytes were then added to the reaction mixture. The results showed that IgA did not interfere with IgG- or IgM-mediated killing of live cells. In this system, killing of >0.9 log (90% of the input inoculum) was mediated by specific IgA alone.

Interaction of isolated immunoglobulin isotypes with purified mononuclear cells or PMNs. Monocyte and PMN populations were purified by differential centrifugation on lymphocyte separation medium and dextran sedimentation. These preparations were tested for their ability to interact with each of the isolated immunoglobulin isotypes, with and without complement, in an opsonophagocytosis experiment. Bacteria were opsonized with specific isotypes and then washed prior to addition of the purified phagocytic cells and complement. In order to ensure complete opsonization of bacteria, we measured the decrease in binding activity (ng of antigen bound/100 μl) for the isolated isotypes after opsonization. In all cases, a measurable reduction in binding was noted; this result indicated adequate coating of the bacteria by specific immunoglobulins. Table 3 shows that in the presence of complement and PMNs, IgG and IgA mediated about equal killing (0.64 and 0.66 log, respectively) of the input inoculum after 60 min of incubation. IgM plus complement and PMNs killed only half as many bacteria (0.23 log) as did IgG or IgA plus complement. Deletion of complement reduced IgG-mediated phagocytic killing to 0.39 log (18% fewer bacteria killed), IgA-mediated killing to 0.24 log (36% fewer bacteria killed), and IgM-mediated killing to 0.02 log. Thus, IgM without complement was not able to facilitate the killing of *P aeruginosa* in the presence of PMNs. The potentiation of IgA-mediated killing by complement was probably due to nonspecific activation of complement by either agglutinated organisms or IgA aggregates on the surface of the bacteria.

Table 3. Mediation of opsonophagocytic killing of IT-1 *P aeruginosa* by purified serum immunoglobulin isotypes, with and without complement (C'), in the presence of isolated peripheral-blood PMNs and mononuclear cells.

Cell type, components added	Log_{10} kill of initial inoculum at 60 min (% killed)*
PMNs	
IgG, C'	0.64 (77)
IgG	0.39 (59)
IgA, C'	0.66 (78)
IgA	0.24 (42)
IgM, C'	0.23 (41)
IgM	0.02 (5)
C'	0
Medium	0.03 (6)
Mononuclear cells	
IgG, C'	0.35 (55)
IgG	0.22 (40)
IgA, C'	0.62 (76)
IgA	0.49 (68)
IgM, C'	0.29 (49)
IgM	0.02 (5)
C'	0
Medium	0

* A value of 0 indicates growth in the tube during a 60-min incubation period.

When mononuclear cells were tested for their ability to kill bacteria coated with the isolated immunoglobulin isotypes, IgA plus complement was found to mediate the best killing (0.62 log, table 3). Without complement, IgA and monocytes killed only 8% fewer bacteria (0.49 log). IgG plus complement and monocytes killed 55% of the input inoculum (0.35 log), while IgM plus complement and mononuclear cells killed 49% (0.29 log). IgM-mediated killing by mononuclear cells was negligible in the absence of complement (0.02 log) whereas IgG and monocytes without complement mediated killing of 40% of the input inoculum (0.22 log).

Discussion

These data represent a detailed analysis of the human immune response to the high-molecular-weight polysaccharide vaccine from IT-1 *P aerugi-*

nosa. We found that a single dose of this vaccine resulted in long-term elevation of levels of antibody specific for the IT-1 polysaccharide determinant. We also found responses to heterologous polysaccharide immunotypes in some vaccinees receiving large doses of the vaccine. In addition, we noted that the ratio of serum immunoglobulins specific for the IT-1 polysaccharide was unaffected by vaccination. Most vaccinees had IgG as the predominant serum antibody, while a few had high levels of IgM antibody. All three of the serum immunoglobulin isotypes interacted with phagocytic cells from peripheral blood to mediate killing of *P aeruginosa*. Complement was required for IgM-mediated phagocytic killing, potentiated IgG-mediated killing, and was not necessary for IgA-mediated killing. IgA did not block opsonophagocytosis mediated by IgG or IgM plus complement.

The association of large doses of vaccine (250 μg) with human responses to heterologous polysaccharide antigens is consistent with previous findings in animals [20]. This association may be due to stimulation, by large antigen doses, of antibodies with a low affinity for IT-1 polysaccharide but a high affinity for heterologous polysaccharide determinants. This result is probably based on the structural similarity of the IT-1 and IT-2 polysaccharide antigenic determinants. Thus, large doses of antigen may stimulate lymphocyte clones with specificity for structurally related polysaccharide determinants. The fact that we were unable to elicit antibodies to heterologous polysaccharide determinants consistently with the IT-1 polysaccharide vaccine suggests that a comprehensive multivalent vaccine to *P aeruginosa* would need to include polysaccharide immunotypes from all clinically important strains.

Previous data on the relative role of IgG and IgM antibodies in promoting opsonophagocytosis of *P aeruginosa* have suggested that IgG opsonins are more effective [1, 21, 22]. We confirmed that observation, utilizing antibody with specificity to the IT-1 polysaccharide. However, the authors of previous studies have not considered the role of serum IgA. Reynolds et al [21] studied the ability of specific secretory IgA and IgG antibodies to promote opsonophagocytic killing of live *P aeruginosa* by human alveolar macrophages. Their studies showed that IgG antibody and alveolar macrophages were effective in killing *P aeruginosa* but that secretory IgA and alveolar macrophages were not. We found that serum IgA and peripheral-blood leukocytes killed a high proportion of the initial inoculum of *P aeruginosa* and that this killing was independent of complement. IgM, however, appeared to have an absolute requirement for complement in mediating opsonophagocytic killing. IgG promoted phagocytic killing in the absence of complement, but levels of killing were higher in the presence of complement.

In experiments testing the interaction of the three major serum immunoglobulin isotypes with isolated mononuclear cells and PMNs, we demonstrated killing of *P aeruginosa* in all cases. The requirement for complement in IgM-mediated killing probably reflects the lack of receptors for IgM on phagocytic cells [23]. With PMNs, IgG best promoted phagocytic killing; with mononuclear cells, IgA was the best mediator. Although our purified IgA preparation may have contained low levels of IgG (up to 3%), it is unlikely that this IgG was responsible for the opsonic activity of the IgA preparation in conjunction with monocytes. IgA consistently mediated opsonic killing with monocytes better than did IgG of >97% purity (table 3). If contaminating IgG in IgA preparations had been responsible for the opsonic killing activity of IgA, we would have expected to see less killing with IgA than with purified IgG. This was not the case. On the other hand, low levels of IgG in IgA preparations may have accounted for the opsonic activity of IgA and PMNs. For IgG and IgA, the level of killing with complement and PMNs was comparable, as was the decrease in killing when complement was deleted. Thus, these data do not indicate whether PMNs and IgA can mediate opsonic killing of *P aeruginosa*. Alternatively, the enhancement of IgA-mediated killing by complement in conjunction with PMNs may have been an artifact of the experimental system used here, which led to nonspecific activation of the complement pathway by IgA-coated organisms. Since PMNs are known to have receptors for C3b [24], the deposition of this complement component on the surface of the cells probably contributed to the enhancement of phagocytic killing of IgA-coated bacteria by PMNs.

The ability of both PMNs and monocytes to kill *P aeruginosa* opsonized with IgG and IgA is probably due to receptors for the Fc portion of these immunoglobulins on human peripheral-blood cells.

Fanger et al [25] showed that both purified PMNs and monocytes express receptors for IgA. These authors and Lawrence et al [23] showed that PMNs and monocytes also express receptors for IgG. In contrast, the latter authors [23] found that monocytes bind IgG1 and IgG3 antibodies but not IgA, whereas PMNs bind these IgG antibodies as well as IgA antibodies. Lowell et al [26] showed that IgA purified from the sera of patients convalescing from infection with group C *Neisseria meningitidis* is able to bind to human monocytes and to mediate antibody-dependent cellular cytotoxicity against *N meningitidis*. This evidence further supports the contention that human monocytes have receptors for serum IgA. Secretory IgA, on the other hand, does not appear to interact effectively with human peripheral-blood cells; Reynolds et al [21] demonstrated this point in experiments with *P aeruginosa*, and Wilson [27] showed that human secretory IgA is not effective in opsonizing *Staphylococcus aureus* for in vitro phagocytic killing. Cooper and Rowley [28] showed that lung clearance of organisms opsonized with immune serum is enhanced in the presence of IgG but not of secretory IgA; this evidence also supports the contention that alveolar macrophages lack receptors for secretory IgA. Our data, however, support the idea that circulating monocytes have receptors for serum IgA. If such receptors are also present on phagocytic cells in the reticuloendothelial system, then elevated serum levels of *P aeruginosa*-specific IgA may be important in protecting the neutropenic host.

The high-molecular-weight polysaccharide vaccine from IT-1 *P aeruginosa* elicits an immune response of long duration. This immune response consists of immunoglobulin isotypes that are all capable of mediating opsonophagocytosis of *P aeruginosa*. IgM-mediated opsonophagocytosis is dependent on complement. In light of these results, we plan to test this vaccine further in phase II human trials, and we hope to develop polysaccharide vaccines from other important serotypes of *P aeruginosa*. The ultimate efficacy of this vaccine in populations at risk will depend on numerous other factors not studied here. However, these results are encouraging because they show that the immune response is long lasting, that the immunoglobulin isotypes induced are appropriate for the killing of live cells, and that vaccine administration is not associated with adverse reactions.

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Further Purification and Characterization of High-Molecular-Weight Polysaccharide from *Pseudomonas aeruginosa*

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Previously published reports on high-molecular-weight polysaccharides from immunotype 1 and 2 of *Pseudomonas aeruginosa* indicated the presence of high levels of mannose in these preparations. This mannose has been found to be due to the presence of a yeastlike mannan in high-molecular-weight polysaccharide preparations. The source of the mannan was found to be the tryptic soy broth used to grow the bacteria. Mannan could be removed from the polysaccharide preparations by chromatography over columns of concanavalin A-Sepharose. The resulting polysaccharides had the same serological reactivity against rabbit antisera and the same immunogenic properties in mice as did the mannan-containing polysaccharides. Comparison of mannan-depleted polysaccharide with preparations of high-molecular-weight polysaccharide obtained from either ultrafiltered tryptic soy broth or a chemically defined medium showed that these polysaccharides were immunologically and chemically similar. Human immune responses to mannan-depleted polysaccharide from the immunotype 1 strain of *P. aeruginosa* were comparable with those previously seen in humans receiving mannan-containing polysaccharides. Thus, we found that *P. aeruginosa* high-molecular-weight polysaccharides prepared in either tryptic soy broth and then subjected to concanavalin A-Sepharose chromatography, ultrafiltered tryptic soy broth, or a chemically defined medium were immunologically and chemically comparable.

The safety and immunogenicity of high-molecular-weight polysaccharide (PS) from immunotype 1 *Pseudomonas aeruginosa* has been established in humans (9). The basis for this trial was the finding that high-molecular-weight PS could be isolated from culture supernates of *P. aeruginosa* and that this material was immunogenic in animals (12, 13) and protected mice from intraperitoneal challenge with live organisms (11, 12). High-molecular-weight PS appears to be an immunogenic, nontoxic form of the lipopolysaccharide (LPS) "O" side chain. A hallmark that distinguishes PS from LPS and LPS O side chains has been the chemical composition of PS. One of these chemical distinctions has been the presence of high levels of mannose in PS preparations, levels not seen in LPS (2, 16). Investigations into the structure of the high-molecular-weight PS from immunotype 1 *P. aeruginosa* led to the identification of a yeastlike mannan in these preparations. This mannan was found by methylation analysis (1, 5) to be a highly branched polymer of alpha (1-2)- and alpha (1,6)-linked mannopyranosyl residues. Exploration of the role of the mannan in the serological and immunogenic activity of high-molecular-weight PS from both immunotype 1 and 2 *P. aeruginosa* led to the finding that mannan was separable from the serologically active and immunogenic components. This report compares the immunochemical properties of immunotype 1 and 2 PS lacking mannan with the previously described properties of PS containing mannan from these strains of *P. aeruginosa*. In addition, we examined the human immune response to mannan-depleted PS from the immunotype 1 strain of *P. aeruginosa*.

MATERIALS AND METHODS

Bacteria. Immunotype 1 and 2 strains of *P. aeruginosa*, originally obtained from M. Fisher, Detroit, Mich., were used throughout the study.

Antigens. For immunochemical analyses and animal studies, high-molecular-weight PS from immunotype 1 and 2 strains of *P. aeruginosa* were prepared from three different media as previously described (9, 12, 13). The media employed were unmodified tryptic soy broth (TSB) with 3% sodium acetate, ultrafiltered tryptic soy broth (UF-TSB) with 3% sodium acetate, prepared by passage of TSB through 10,000-molecular-weight cutoff membranes, and the chemically de-

fined medium (CDM) described by Terleckyj et al. (15). To remove the medium mannan component, materials obtained from TSB cultures were further purified by chromatography over columns of concanavalin A covalently linked to Sepharose (ConA-Sepharose) (Pharmacia Fine Chemicals, Piscataway, N.J.) in buffer containing 0.1 M acetate, 0.15 M NaCl, 1 mM each $CaCl_2$, $MgCl_2$, and $MnCl_2$, and 0.1% Merthiolate, pH 6.5.

Human immunizations. High-molecular-weight PS from immunotype 1 *P. aeruginosa* was prepared from culture supernates of bacteria grown in unmodified TSB then depleted of mannan by ConA-Sepharose chromatography. Two separate lots (number 3 and 4) were made, and then each was bottled by the Massachussetts State Biological Laboratories for injection into humans as previously described (9). These materials were tested for safety and toxicity in laboratory animals as outlined by the Food and Drug Administration regulations (Title 21, Section 610.11). Informed consent was obtained from all volunteers before the administration of the vaccine. Approval for this study was obtained from the Committee for the Protection of Human Subjects from Research Risks, Brigham and Women's Hospital.

Mitogenesis assays. Mitogenic activity of PS preparations on mouse splenocytes was assayed as previously described (10). Purified ConA for use in these experiments was obtained from Pharmacia Fine Chemicals. Stimulation indices were calculated by dividing the counts per minute of [^{3}H]thymidine incorporated into stimulated cultures by the counts per minute incorporated into unstimulated cultures.

Serological assays. Antigen preparations were tested for serological identity in Ouchterlony immunodiffusion gels, as previously described (12, 13). These gels were also used to assess the reactivity of antigen preparations with ConA. Solutions of ConA (5 mg/ml) were substituted for serum when indicated. Analysis of antibody binding levels in mouse and human sera was accomplished using a radioactive antigen binding assay as previously described (8, 9). Statistical comparison of antibody titers after immunization of animals and humans was done with a *t* test. Testing of the opsonic activity in human serum, pre- and post-immunization, was done using an opsonophagocytic assay that was previously described (9). Determination of antibodies to *Candida albicans* mannan was kindly performed by Michael Lew using an enzyme-linked immunosorbent assay (2).

Mouse immunizations. C3H/ANF mice were immunized by injection with 10 μg of antigen in 0.5 ml of saline. Mice were bled before and 7 days after the immunization from the retroorbital plexus while under ether anesthesia. These sera were then tested in the radioactive antigen binding assay. Mouse data are expressed as increases in the antigen binding capacity (counts per minute bound × specific activity of antigen) per 100 μl of serum minus the preimmune antigen binding capacity.

Chemical components. Determination of the protein level in PS preparations was done by the method of Lowry et al. (6). Determination of nucleic acids was done as previously described (12, 13). Determination of the 2-keto-3-deoxyoctulosonic acid concentrations was done by the method of Osborn (7). Quantitative analysis of the monosaccharide constituents in PS preparations was done using the alditol acetate method of Sawardeker et al. (14). Analysis was carried out on a column (2 mm by 3 ft [ca. 91 cm]) of SP 2340 (Supelco, Inc., Bellefonte, Pa.) in a Packard 421 gas-liquid chromatograph. Analysis conditions were as follows: nitrogen carrier gas flow, 20 ml/min; initial temperature, 160°C held for 3 min, temperature rise of 5°C/min to a final temperature of 255°C; injector temperature, 210°C, detector temperature, 260°C. Sugars were identified based on their retention times (as compared with authentic standards) and cochromatography with known standards, except for the dideoxyhexoseamines. These were identified based on the retention times of known dideoxyhexoseamines found in the *P. aeruginosa* LPS (1) and the capsular PS of *Bacteroides fragilis* 23745 (3). The former materials were prepared by us as previously described (12, 13), and the latter material was kindly supplied by Dennis L. Kasper, Channing Laboratory. Quantitation was performed using a Hewlett Packard 3388 integrator. The total amounts of each sugar were calculated by the integrator, which had been previously programmed with the response factors to individual monosaccharides by using known amounts of authentic standards. The total carbohydrate content of each antigen preparation was determined by dividing the sum of the total amount of the sugars identified by the total amount of antigen injected into the gas-liquid chromatograph. The latter amount was determined by use of an internal standard of inositol added to the antigen preparation before the preparation of the alditol acetate derivative.

RESULTS

Comparison of mannan-containing and mannan-depleted PS. After identification of a mannan component in high-molecular-weight PS preparations, we prepared mannan-depleted PS for immunochemical analyses from *P. aeruginosa* immunotype 1 and 2 and compared the serological properties of these two antigens. Figure 1 shows the immunodiffusion reaction of immunotype 1 and 2 PS before and after chromatography on ConA-Sepharose against rabbit antisera raised to whole organisms and against solutions of ConA. This chromatography step separated the mannan component from the serologically active component in high-molecular-weight PS preparations. As a test for the potential source of the mannan in the PS preparations, we looked at the reactivity of unmodified TSB media with solutions of ConA. We found that TSB contained a material giving a precipitin line in gel diffusion against solutions of ConA (Fig. 1).

Table 1 compares the immunogenicity of 10 μg of the mannan-containing and mannan-depleted high-molecular-weight PS preparations in inbred C3H/ANF mice. Both mannan-containing and mannan-depleted immunotype 1 and 2 PS induce significant ($P < 0.01$) increases in binding antibody against homologous PS. The mannan-depleted immunotype 1 and 2 PS were also cross-immunogenic in outbred mice (not

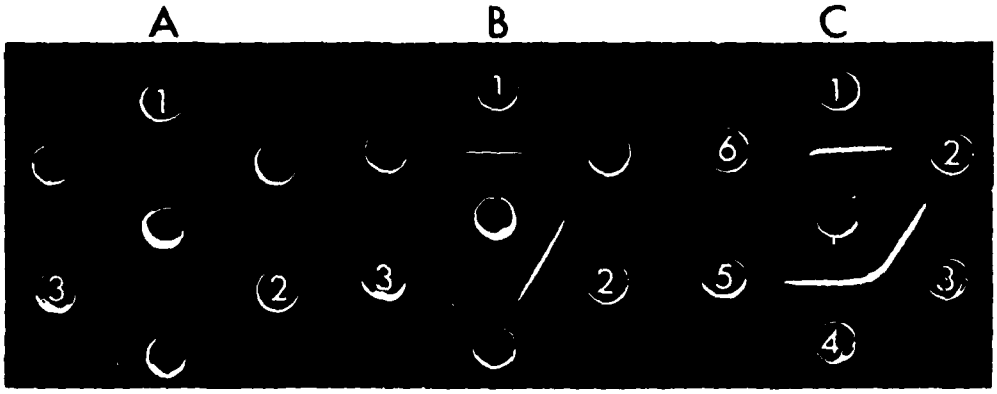

FIG. 1. Immunodiffusion analysis of the reaction of antisera raised to whole immunotype 1 and 2 *P. aeruginosa* organisms and solutions of ConA against preparations of high-molecular-weight PS either containing mannan or depleted of mannan by ConA-Sepharose chromatography. (A) Center well, antiserum to immunotype 1 *P. aeruginosa*, 20 μl; outer wells—(1) mannan-containing immunotype 1 PS, 1 mg/ml; (2) immunotype 1 PS after ConA-Sepharose chromatography, 1 mg/ml; (3) purified mannan (eluted from ConA-Sepharose column after binding), 1 mg/ml. (B) Center well, antiserum to immunotype 2 *P. aeruginosa*, 20 μl; outer wells—(1) mannan-containing immunotype 2 PS, 1 mg/ml; (2) immunotype 2 PS after ConA-Sepharose chromatography, 1 mg/ml; (3) purified mannan, 1 mg/ml. (C) Center well, ConA, 5 mg/ml, 20 μl; outer wells—(1) immunotype 1 PS before ConA-Sepharose chromatography, 1 mg/ml; (2) immunotype 1 PS after ConA-Sepharose chromatography, 1 mg/ml; (3) purified mannan, 1 mg/ml; (4) immunotype 2 PS before ConA-Sepharose chromatography, 1 mg/ml; (5) immunotype 2 PS after ConA-Sepharose chromatography, 1 mg/ml; (6) tryptic soy broth, 20 μl.

shown), as has been previously reported for the mannan-containing PS (8).

We next prepared high-molecular-weight PS from UF-TSB and from the CDM of Terleckyj et al. (15). The yield of PS from UF-TSB was quite low (0.5 mg/liter) compared with the yield from regular TSB after mannan removal (5 mg/liter) or from CDM (4 mg/liter), making the use of UF-TSB for routine production of PS impractical. High-molecular-weight PS obtained from both UF-TSB and CDM gave reactions of identity in immunodiffusion gels with both mannan-containing and mannan-depleted PS preparations. These materials were also comparably immunogenic in mice (Table 1). The PS preparation from the CDM did not react with ConA in immunodiffusion gels, but PS from UF-TSB had a faint line versus solutions of ConA (not shown). This reactivity could be removed by ConA-Sepharose chromatography and indicates that ultrafiltration of the TSB did not successfully remove all of the media mannan component. However, these results do indicate that preparation of high-molecular-weight PS from either UF-TSB or CDM resulted in an antigen that was immunologically similar to previously described high-molecular-weight PS preparations.

Chemical composition and monosaccharide constituents of mannan-containing and mannan-depleted PS. Table 2 shows the chemical composition of high-molecular-weight PS obtained from the various media described above. All of these PS preparations contained similar levels of contaminating protein and nucleic acids, and all were negative for 2-keto-3-deoxyoctulosonic acid in the thiobarbituric acid assay. Chromatography of mannan-containing PS over ConA-Sepharose columns did not affect the protein content, indicating that ConA did not leach from these affinity gels into the PS preparations. The total amount of each preparation identifiable as carbohydrate by quantitative gas-liquid chromatography was similar for all preparations.

Table 3 shows the monosaccharide constituents of PS obtained from immunotype 1 and 2 *P. aeruginosa*. These materials were obtained from TSB before and after ConA-Sepharose chromatography, from UF-TSB, or from the CDM. As expected, PS obtained from TSB before ConA-Sepharose chromatography had a high level of mannose. PS from UF-TSB had a reduced content of mannose compared with PS isolated from TSB and not chromatographed. (The mannose content in PS from UF-TSB could be reduced even further by ConA-Sepharose chromatography.) The PS preparations obtained from the CDM had a similar quantitative and qualitative monosaccharide composition to mannan-depleted PS. This fact indicates that ConA-Sepharose chromatography yielded PS that was composed principally of bacterial products. Interestingly, all preparations of high-molecular-

TABLE 1. Immunogenicity of 10 μg of various high-molecular-weight PS preparations in C3H/ANF mice

Immunogen	Prepared in/by:	Increase over preimmune serum in antigen binding capacity[a] vs homologous PS
Immunotype 1 PS	TSB	55 ± 39[b]
	After ConA-Sepharose	66 ± 30
	UF-TSB	51 ± 20
	CDM	72 ± 22
Saline		0
Immunotype 2 PS	TSB	150 ± 22
	After ConA-Sepharose	140 ± 28
	UF-TSB	147 ± 33
	CDM	136 ± 18
Saline		0

[a] Antigen binding capacity is counts per minute of antigen bound by 100 μl of serum × the specific activity of the radiolabeled antigen.

[b] Average of four mice ± standard deviation.

weight PS studied here contained high levels of galactose and arabinose, two monosaccharides not found in *P. aeruginosa* LPS (2, 16).

Safety and immunogenicity of mannan-depleted immunotype 1 PS in humans. The safety and immunogenicity of immunotype 1 PS depleted of mannan by ConA-Sepharose chromatography was assessed in eight humans. Toxicity testing in mice and guinea pigs and pyrogenicity testing in rabbits were performed by the Massachusetts State Biological Laboratories on two lots (3 and 4) of mannan-depleted immunotype 1 PS, as described previously (9). Before administration of this material to humans, we tested the PS for mitogenicity on murine splenocytes. This was to determine whether residual ConA was present in the vaccine. Table 4 shows that mannan-depleted lots 3 and 4 both had less mitogenic activity than mannan-containing immunotype 1 PS. This suggests that the mannan may have some mitogenic properties. At the highest doses of mannan-depleted PS tested (500 μg/ml) there was little to no mitogenic response. ConA was, of course, a potent mitogen for murine spleen cells, and addition of high levels (500×) of PS to mitogenic doses of ConA did not affect the mitogenic properties of ConA. Thus, no detectable ConA could be found in the PS preparations by the murine splenocyte mitogenesis assay.

Five humans received 100 μg of lot 3 immunotype 1 PS, and three received 100 μg of lot 4. The geometric mean antibody levels preimmunization and at 14 and 28 days post-immunization are shown in Table 5. A significant ($P < 0.001$) increase in binding antibody was observed in vaccinates at both 14 and 28 days post-immunization. No reaction to the vaccine up to 72 h post-immunization was noted in any vaccinate either locally or systemically, except a slightly sore and tender arm at the injection site in one of eight vaccinates, confirming our previous observation (9) that reactions are unusual with *P. aeruginosa* PS vaccines. Increases in opsonic titers of fourfold or greater were observed for seven of the eight vaccinates (Table 5). Thus, the mannan-depleted high-molecular-weight PS possessed similar immunogenic properties in humans as had previously described PS preparations. In addition, this vaccine elicited only a mild local reaction in one of eight vaccinates.

Response of humans to *C. albicans* mannan. Because 42 persons had been previously immunized with immunotype 1 PS containing mannan (9), we selected 10 sera from persons given this vaccine and tested their antibody responses to *C. albicans* mannan, as described previously (4). This mannan is structurally identical to the mannan found in our PS preparations. No increase in *enzyme-linked immunosorbent assay* binding titers was seen in the pre- and post-vaccination sera of persons receiving mannan-containing PS vaccines, indicating that the mannan component was not immunogenic in these vaccinates.

DISCUSSION

The identification of a mannan component, likely from the medium, in high-molecular-

TABLE 2. Chemical composition of various high-molecular-weight PS preparations

Preparation	%			
	Protein	Nucleic acid	KDO[a]	Carbohydrate
Immunotype 1 PS from				
TSB	2.7	0.7	ND[b]	73
After ConA-Sepharose	1.8	0.6	ND	71
UF-TSB	0.1	0.3	ND	82
CDM	0.4	0.7	ND	78
Immunotype 2 PS from				
TSB	3.7	0.6	ND	75
After ConA-Sepharose	3.4	0.4	ND	73
UF-TSB	2.0	0.8	ND	77
CDM	1.6	0.9	ND	79

[a] Percentage is calculated as total amount of material detected divided by total amount of material measured. Unaccounted for material is moisture (13).

[b] KDO, 2-Keto-3-deoxyoctulosonic acid; ND, none detected

TABLE 3. Monosaccharide constituents of various high-molecular-weight PS preparations

Preparation	Monosaccharide[a] (% total identified)						
	Rham	Ara	Xyl	Man	Gal	Glu	Dideoxy[b]
Immunotype 1 PS from:							
TSB	3.2	5.8	ND[c]	54.4	17.6	7.2	ND
After ConA-Sepharose	24.5	24.0	3.1	ND	30.0	9.0	11.0
UF-TSB	23.2	20.1	4.5	tr	30.9	17.0	7.3
CDM	22.4	20.9	tr	ND	31.1	15.4	10.2
Immunotype 2 PS from:							
TSB	4.8	5.0	7.3	45.1	22.5	10.7	3.7
After ConA-Sepharose	1.2	7.2	tr	ND	45.6	26.1	20.0
UF-TSB	2.8	8.2	2.7	tr	40.4	22.0	24.0
CDM	2.4	7.9	2.1	ND	41.2	23.1	23.3

[a] Rham, rhammose; ara, arabinose; xyl, xylose; man, mannose; gal, galactose; glu, glucose; dideoxy, dideoxyhexoseamine.

[b] Amount of dideoxyhexoseamine calculated from area of glucoseaminitol hexaacetate standard. The lack of a dideoxyhexoseamine standard prohibits determining an accurate amount.

[c] ND, None detected.

weight PS preparations from *P. aeruginosa* necessitated a determination of the role of this material in the serological activity and immunogenicity of PS. We found that removal of mannan by ConA-Sepharose chromatography resulted in a material that contained similar serological activity and immunogenicity as those of mannan-containing PS. The mannan-depleted PS from immunotype 1 was also immunogenic in humans, inducing significant increases in binding and opsonic antibody after immunization with a single 100-μg dose. There were no serious untoward reactions to the vaccine among eight vaccinates. Preparation of high-molecular-weight PS in a CDM resulted in a material immunologically identical and chemically similar to PS prepared in TSB and depleted of mannan by ConA-Sepharose chromatography. Thus, these two methods likely yield a PS preparation consisting solely of bacterial products.

TABLE 4. Mitogenic stimulation indices of immunotype 1 *P. aeruginosa* PS human vaccines on murine splenocytes

Immunotype 1 vaccine	Dose (μg/ml)	Stimulation index
Lot 3	500	0.7
	250	0.5
	100	0.8
	50	0.7
Lot 4	500	1.5
	250	0.9
	100	1.2
	50	1.3
Lot 3 before mannan depletion	500	3.7
	250	3.9
	100	2.0
	50	1.7
ConA	1	4.9
Plus lot 3[a]	500	4.6
Plus lot 4[a]	500	5.4

[a] ConA at 1 μg/ml plus indicated PS vaccine at 500 μg/ml.

The use of ultrafiltered or dialyzed media for the production of bacterial vaccines is generally employed to avoid media contamination of the final product. Out initial attempts to produce PS in UF-TSB, before preparing any material for human use, showed that bacterial growth and antigen production in UF-TSB were severely reduced. Also, before human studies, we isolated high-molecular-weight components of TSB and could not identify any carbohydrate in this fraction. The presence of mannan from the medium in our high-molecular-weight PS preparations was, therefore, unexpected. However, because PS are prepared from large culture volumes (20 to 30 liters), low levels of mannan (<1%) in TSB could go undetected but end up in high proportion in the final PS product, if the mannan copurifies with the PS, which appears to be the case. Although UF-TSB is a poor medium for PS production, the CDM seems to be adequate for antigen production and is the preferable medium for producing high-molecular-weight PS. Recent attempts to produce PS from both ultrafiltered Todd-Hewitt broth or ultrafiltered Columbia broth indicate that these media may be superior to the CDM because of greater yields of material.

Particularly important is the finding of chemical similarity among PS depleted of mannan by ConA-Sepharose chromatography, PS obtained from UF-TSB, and PS obtained from the CDM. Thus, the components of PS in these preparations are likely to be bacterial products and not media or other contaminants. High-molecular-

TABLE 5. *Antibody levels and opsonic titers in sera from eight human vaccinates before and after a 100-μg dose of immunotype 1 PS*[a]

Serum sample	Antibody level[b] (μg/ml)	Opsonic titer[c]
Preimmune	6.02 ± 1.5 (3.9–14.1)	2.6 ± 0.8 (2–8)
Day 14	39.9 ± 2.8[d] (8.9–158.9)	26.3 ± 2.4[d] (8–128)
Day 28	48.8 ± 3.0[d] (11.5–180.7)	26.3 ± 3.3[d] (8–128)

[a] Five vaccinates received lot 3 and three received lot 4.
[b] Determined in the radioactive antigen binding assay as described previously (9). Reported as $\log_{10}$ geometric mean ± standard deviation. Range is shown within parentheses.
[c] The titer is the reciprocal of the serum dilution showing ≥90% kill of the input inoculum as described previously (9). Results are expressed as $\log_2$ geometric mean ± standard deviation. Range is shown within parentheses.
[d] $P < 0.001$.

weight PS obtained from TSB after a ConA-Sepharose chromatography step, from UF-TSB, or from the CDM, all contained the monosaccharides galactose and arabinose. These two monosaccharides are absent from LPS (2, 16). Thus, high-molecular-weight PS remains chemically distinct from both intact LPS and O side chains by virtue of its monosaccharide constituents. Since galactose and arabinose appeared in all PS preparations made here, they must be bacterial components of PS preparation. Whether galactose and arabinose are covalently linked to the monosaccharides shared by PS and LPS O side chains remains to be seen. Attempts to separate the galactose and arabinose components from the serologically active monosaccharides that are shared between LPS and PS thus far have been unsuccessful. However, it is possible that an arabinogalactan-like molecule is present in the PS preparations, along with a high-molecular-weight form of the LPS O side chain. Structural studies are now under way to determine whether this is the case. Currently, we are able to prepare nontoxic PS that is immunologically active in humans, giving rise to binding and opsonic antibodies directed at the serotype determinant located on the O polysaccharide side chain of the LPS.

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Immunization with *Pseudomonas aeruginosa* High-Molecular-Weight Polysaccharides Prevents Death from *Pseudomonas* Burn Infections in Mice

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High-molecular-weight polysaccharides from the extracellular slime of *Pseudomonas aeruginosa* were evaluated as immunogens in *Pseudomonas* burn infections in mice. Immunization with immunotype 1 or 2 polysaccharides induced a strong immunotype-specific and weak cross-reactive antibody response but protected mice against burn infections caused by either immunotype. Passive protection was provided by rabbit antiserum to immunotype 1 polysaccharide against burn infection by the homologous organism. *Pseudomonas* high-molecular-weight polysaccharides are potentially effective vaccines in burn infections.

High-molecular-weight polysaccharides isolated from culture supernatants or from extracellular slime of *Pseudomonas aeruginosa* appear to share immunotype determinants with the lipopolysaccharide O side chain (7–9). The best-characterized of these polysaccharides are from *P. aeruginosa* immunotypes 1 (8) and 2 (7), designated It-1 and It-2, of the Fisher-Devlin-Gnabasik system (2). The It-1 and It-2 polysaccharides induce type-specific and cross-reactive antibodies in mice and rabbits (4) and are immunogenic in humans (5; G. B. Pier, unpublished data). Antibodies to both polysaccharides are opsonic and provide cross-protection against intraperitoneal challenges in mice (4, 6, 7).

Since patients with extensive burns are particularly susceptible to life-threatening *Pseudomonas* infections, they are prime candidates for specific immunoprophylaxis or immunotherapy. A murine *Pseudomonas* burn infection model (3, 10) closely mimics human *Pseudomonas* burn wound sepsis and avoids some of the unphysiological aspects of massive intraperitoneal challenges. We used this model to evaluate the protective efficacy of the It-1 and It-2 polysaccharide vaccines in *P. aeruginosa* burn infections.

Six-week-old female C3H/FeJ mice (Jackson Laboratories, Bar Harbor, Maine) were immunized on days 0, 5, and 10 with 50-μg intraperitoneal injections of high-molecular-weight polysaccharide obtained from the culture supernatant of *P. aeruginosa* It-1 (8) or It-2 (7) and suspended in normal saline. Control mice received intraperitoneal injections of bovine serum albumin (BSA) according to an identical dose schedule. On day 15, the mice were anesthetized with methoxyflurane (Pitman-Moore, Inc., Washington Crossing, N.J.), subjected to an 11-s alcohol flame burn (2.5 by 2.5 cm), and injected subcutaneously at the burn site with seven 10-fold dilutions of washed log-phase It-1 or It-2 bacteria. Deaths were recorded for 7 days, and the 50% lethal dose (LD_{50}) of bacteria was determined by the method of Spearman-Karber (1). Blood was obtained from the retro-orbital venous plexus of anesthetized mice on days 0 and 15, and serum antibodies reactive with *P. aeruginosa* It-1 and It-2 polysaccharides were quantified by a radioactive antigen-binding assay (4).

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Immunized mice demonstrated an immunotype-specific serum antibody response (Table 1). In addition, there was a small but significant antibody response to It-2 polysaccharide in mice immunized with It-1 polysaccharide, and vice versa (Table 1). The burn injury itself apparently had little or no effect on the immunogenicity of It-1 polysaccharide, as indicated by nearly identical serum antibody responses in recently burned and unburned mice immunized with a single 50-μg intraperitoneal injection of this vaccine (Table 2). Immunization with three 50-μg doses of It-1 polysaccharide protected mice against subsequent It-1 burn infections, as indicated by a greater than 4 log increase in the LD_{50} of the It-1 challenge strain compared with its LD_{50} in BSA-immunized control mice (Table 3). Immunization with It-1 polysaccharide also produced cross-protection against It-2 burn infections, as indicated by a 1 to 2 log increase in the LD_{50} of It-2 organisms. Similarly, immunization with It-2 polysaccharide protected against both It-2 and It-1 burn infections, as evidenced by 2 to 3 log and 4 to 5 log increases in LD_{50}, respectively (Table 3). It-2 immunization appeared to provide somewhat greater protection against both homologous and heterologous challenges than did immunization with It-1 polysaccharide.

The intravenous administration of 0.2 ml of rabbit antiserum to It-1 polysaccharide (8) 18 h before burn infection resulted in a 2 to 3 log increase in the LD_{50} of the It-1

TABLE 1. Serum antibody responses of C3H/FeJ mice to immunization with *P. aeruginosa* It-1 and It-2 high-molecular-weight polysaccharides

Immunization[a]	Serum antibody concn (μg/ml)[b]			
	It-1		It-2	
	Day 0	Day 15	Day 0	Day 15
It-1	4.1 ± 0.4	35.2 ± 9.3[c]	3.8 ± 0.2	4.4 ± 0.2[c]
It-2	4.0 ± 0.2	5.2 ± 0.5[c]	10.5 ± 8.4	64.1 ± 26.4[c]

[a] Fifty micrograms were injected intraperitoneally on days 0, 5, and 10.

[b] Radioactive antigen binding assay; mean ± standard deviation; five mice per group.

[c] Significant increase compared with preimmunization level; $P \leq 0.02$ based on *t*-test on paired samples.

TABLE 2. Effect of burn injury on serum antibody responses of C3H/FeJ mice to immunization with *P. aeruginosa* It-1 high-molecular-weight polysaccharide[a]

Group	Serum antibody concn (μg/ml)[b]	
	Day 5	Day 10
Unburned	10.3 ± 5.8	10.8 ± 4.8
Burned	9.3 ± 5.6	11.2 ± 6.4

[a] Groups of 10 mice received 50 μg of It-1 polysaccharide intraperitoneally 24 h after undergoing an 11-s flame burn (2.5 by 2.5 cm) or no injury.

[b] Radioactive antigen-binding assay. Data expressed as mean ± standard deviation. Antibody levels of unimmunized mice were <2.0 μg/ml.

challenge strain compared with that observed in control mice which had received nonimmune serum (Table 4). Passive protection was somewhat less than that produced by active immunization (Table 3).

Thus, despite limited cross-reactive antibody responses induced in C3H/FeJ mice by *P. aeruginosa* It-1 and It-2 high-molecular-weight polysaccharides, cross-protection was comparable to homologous protection in the mouse burn infection model. The possibility exists that It-2 polysaccharide is somewhat more immunogenic than It-1 polysaccharide, as judged by both antibody levels and degree of protection against bacterial challenges. These findings are similar to those previously reported for CD-1 mice subjected to intraperitoneal infections (4).

Our data establish the efficacy of active immunization with It-1 and It-2 high-molecular-weight polysaccharides and passive immunization directed toward It-1 polysaccharide in *Pseudomonas* burn wound sepsis in mice. To the extent that the murine model reproduces human burn wound sepsis and that antibody responses to It-1 and It-2 polysaccharides reflect those achievable in humans (5; G. B. Pier, unpublished data), effective immunoprophylaxis against *Pseudomonas* burn infections with these and other immunotype-specific high-molecular-weight polysaccharide vaccines appears feasible.

TABLE 3. Type-specific and cross-protection against *P. aeruginosa* burn wound sepsis in C3H/FeJ mice after active immunization with It-1 and It-2 polysaccharides

Challenge strain	Immunization[a]	LD_{50} of challenge strain (log_{10} CFU)[b]
It-1	BSA	3.64
	It-1	8.04[c]
	It-2	8.26[c]
It-2	BSA	5.15
	It-1	6.94[c]
	It-2	7.54[c]

[a] Mice received 50 μg of high-molecular-weight polysaccharide or BSA intraperitoneally on days 0, 5, and 10.

[b] On day 15, seven 10-fold dilutions of washed log-phase bacteria were injected at the site of a fresh 11-s flame burn (2.5 by 2.5 cm). Five mice were used at each dilution, deaths were recorded for 7 days, and the LD_{50} ± 95% confidence interval was determined by the method of Spearman-Karber (1).

[c] Significant protection (nonoverlapping LD_{50} ± 95% confidence interval) compared with BSA-immunized controls.

TABLE 4. Protection against *P. aeruginosa* It-1 burn wound sepsis in C3H/FeJ mice passively immunized with type-specific high-molecular-weight polysaccharide antiserum

Immunization[a]	LD_{50} of challenge strain (log_{10} CFU)
Normal serum	4.0
It-1 antiserum	6.8[b]

[a] Mice received 0.2 ml of normal rabbit serum or It-1 polysaccharide antiserum intravenously 18 h before undergoing an 11-s flame burn (2.5 by 2.5 cm) followed by subcutaneous inoculation of the fresh burn site with seven 10-fold dilutions of the It-1 challenge strain. Five mice were used at each dilution, deaths were recorded for 7 days, and the LD_{50} ± 95% confidence interval was determined by the method of Spearman-Karber (1).

[b] Significant protection (nonoverlapping LD_{50} ± 95% confidence interval) compared with control animals that received normal rabbit serum.

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The experiments reported herein were conducted according to the principles set forth in the *Guide for Care and Use of Laboratory Animals*, Institute of Animal Resources, National Research Council, U.S. Department of Health, Education and Welfare publication no. (NIH) 74-23, U.S. Department of Health, Education and Welfare, Washington, D.C.

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Immunochemical Characterization of High-Molecular-Weight Polysaccharide From Fisher Immunotype 3 *Pseudomonas aeruginosa*

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A high-molecular-weight polysaccharide (PS) was isolated from the culture supernatant of a Fisher immunotype 3 (IT-3) strain of *Pseudomonas aeruginosa*. Consistent with previously reported findings for IT-1 and IT-2 PS, the preparation of IT-3 PS was found to be an immunogenic, nontoxic form of the O polysaccharide side chain on the lipopolysaccharide (LPS). The IT-3 PS was mainly carbohydrate in composition. It was serologically and chemically identical to LPS O side chain, but distinct from that structure in molecular size and immunogenicity. The IT-3 PS was nontoxic in mice and guinea pigs, nonpyrogenic in rabbits, and >1,000-fold less reactive than IT-3 LPS in gelation of the *Limulus* amoebocyte lysate. Preliminary analyses by gas-liquid chromatography and ^{13}C nuclear magnetic resonance have established the structural identity of IT-3 high-molecular-weight PS and the IT-3 O side chain. IT-3 PS was immunogenic in rabbits and mice. After active immunization, mice were protected against *P. aeruginosa* IT-3 intraperitoneal infection and burn wound sepsis. IT-3 PS also elicited protection against challenge with an IT-5 strain of *P. aeruginosa*, indicating that low-level contamination of the IT-3 PS with IT-3 LPS was not responsible for the immunogenic activity. These findings demonstrate the feasibility of preparing nontoxic immunogenic IT-3 PS capable of eliciting serotype-specific protective antibodies, employing methods similar to those previously described for the isolation of PS from other *P. aeruginosa* immunotypes.

Investigation of immunity to *Pseudomonas aeruginosa* disease indicates an important role for antibody directed at the serotype or immunotype determinant located on the O polysaccharide portion of the lipopolysaccharide (LPS) (18, 19). Induction of type-specific immunity in animals by either active or passive immunization results in protection from lethal challenge (1, 14, 15). Similarly, protective immunity in humans at risk for *P. aeruginosa* infections has been most closely correlated with type-specific antibody (18, 19). An immunogenic and nontoxic preparation that engendered long-lasting antibodies to the immunotype antigens of *P. aeruginosa* would represent a potentially effective human vaccine.

Earlier attempts to produce this type of immunity by utilizing LPS preparations were hampered by the toxicity of this molecule (10, 24). The recent isolation of a high-molecular-weight polysaccharide (PS) fraction from culture supernatants of *P. aeruginosa* (12, 13, 15) has resulted in preparations that contain the serotype determinant in an immunogenic but nontoxic form. Studies involving the Fisher immunotype 1 (IT-1 and IT-2) strains of *P. aeruginosa* have shown that high-molecular-weight PS preparations elicit type-specific antibody in animals (14, 15) as well as in humans (12, 17; G. B. Pier, manuscript in preparation). Because of the conditions utilized to purify high-molecular-weight PS, some of which subject the PS to highly nonphysiological temperatures and pHs, it is necessary to define the immunochemical properties of PS prepared from the different Fisher immunotypes. In this report, we characterize some of the biochemical, immunological, and protective properties of high-molecular-weight PS prepared from a Fisher IT-3 strain of *P. aeruginosa*.

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MATERIALS AND METHODS

Bacterial strains. High-molecular-weight PS was recovered from the culture supernatant of a clinical isolate of *P. aeruginosa* (strain PBBH 12136-80) that was characterized as a Fisher IT-3 strain (4). It was established in our laboratory that this strain gave high yields of the desired antigen. For some experiments, we also utilized the Fisher IT-5 strain, obtained originally from M. W. Fisher, Parke, Davis & Co., Detroit, Mich.

Preparation of IT-3 PS, LPS, and O polysaccharide side chains. High-molecular-weight PS was prepared as previously described (12, 13, 15). The organisms were grown in 14 liters of the chemically defined medium of Terleckyj et al. (22) in an LSL Biolafitte 20-liter fermentor. The LPS was extracted from lyophilized cells of this IT-3 strain by the phenol-water method of Westphal et al. (23) and purified as previously described (15, 16). O polysaccharide side chains were derived from the LPS by hydrolysis in 1% acetic acid at 95°C for 6 h. The lipid A precipitate was removed by centrifugation, and the supernatant was dialyzed against deionized water and lyophilized.

Rabbit antisera. Antisera were raised in rabbits to whole organisms and to high-molecular-weight PS, using the immunization schedule previously described (16).

Serological methods. Ouchterlony immunodiffusion analyses were performed as previously described (16). Measurement of antibody to the IT-3 high-molecular-weight PS was done by utilizing a radioactive antigen binding assay as described previously (12, 15). Preparation of the intrinsically ^{14}C-labeled IT-3 PS from a minimal medium was as described previously (15).

Chemical analyses. Protein was assayed by the method of Lowry et al. (7). Nucleic acid was determined by absorption

at 254 nm, using salmon sperm DNA as a standard. 2-Keto-3-deoxyoctulosonic acid was assayed by the method of Osborn (8). Lipids were assayed by gas-liquid chromatography of fatty acid methyl esters as described previously (15). Gelation of the *Limulus* amoebocyte lysate was performed in single vial tubes (Mallinckrodt Chemicals, St. Louis, Mo.) according to the manufacturer's instructions.

Monosaccharide analysis. Because of the reported occurrence of unique monosaccharide residues in the O side chain isolated from strains of *P. aeruginosa* that are serologically identical to the Fisher IT-3 strain (5, 6), we developed conditions to attempt to identify these residues in our preparations. Portions of high-molecular-weight PS and O polysaccharide antigens were reduced twice with water-soluble carbodiimide and $NaBH_4$ by the method of Taylor and Conrad (21). Both unreduced and reduced materials (2 mg) were then hydrolyzed with 100 μl of aqueous hydrogen fluoride (HF) (49% in water) in polypropylene tubes at room temperature for 24 h. The HF was removed under vacuum in the presence of NaOH, the sample was redissolved in 200 μl of water, and then one half was removed for further hydrolysis in 2 M HCl at 100°C for 3 h. The two samples were then made to 1 M ammonia with ammonium hydroxide and derivatized to the alditol acetates by the method of Blakeney et al. (2). The samples were analyzed on a Hewlett-Packard 5880A gas-liquid chromatograph equipped with a 25-ft (7.62-m) Silar 10C capillary column (Alltech Associates, Deerfield, Ill.). Detection was with flame ionization detectors. A split ratio of 20:1 was employed. Conditions for analysis were a column pressure of 20 lb/in^2 and an initial temperature of 230°C for 5 min, followed by a rise of 10°C/min to 250°C for a further 10 min. The injector temperature was 250°C, and the detector temperature was 260°C.

Molecular size determinations. The apparent molecular sizes of the IT-3 PS and the O polysaccharide side chain were determined by high-performance liquid chromatography as previously described (13).

Animal studies. The toxicity in mice and guinea pigs of the IT-3 PS was determined as described previously (12). Rabbit pyrogenicity was also assessed as previously described (12). Active immunization of C3H/FeJ mice (Jackson Laboratories, Bar Harbor, Maine) for antibody studies was performed as previously described (11). Blood was obtained pre- and post-immunization from the retroorbital plexus of ether-anesthetized animals, and serum antibody was assayed in the radioactive antigen binding assay described above. Active and passive immunizations of CD-1 mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.), and subsequent intraperitoneal (i.p.) challenge with a dose of live organisms sufficient to kill 90 to 100% of nonimmune mice, were performed as described previously (14). Immunization and challenge of C3H/FeJ mice, utilizing the burned mouse model of Stieritz and Holder (20) as modified by Pavlovskis et al. (9), was also done to assess the protective capacity of IT-3 PS. The 50% lethal inoculum (LD_{50}) ± 95% confidence interval of the challenge organism was determined in immunized and unimmunized control mice by the method of Spearman and Karber, as described by Finney (3).

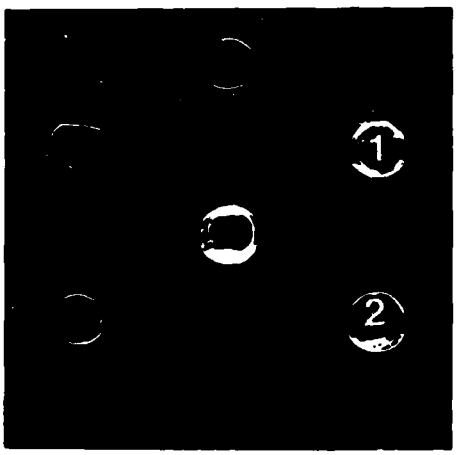

FIG. 1. Reaction of identity between IT-3 high-molecular-weight PS (1) and O side chains (2) against rabbit antiserum raised to whole organisms (center well).

TABLE 1. Properties of peaks found by gas-liquid chromatography analyses of IT-3 PS and O side chains

PS state[a]	Hydrolysis conditions[b]	R_{glu} in peaks found in:	
		IT-3 PS	O side chains
Unreduced	HF	2.07	2.07
Reduced	HF	1.03	1.03
		2.07	2.07
Unreduced	HF-HCl	2.07	2.07
Reduced	HF-HCl	2.07	2.07
Authentic DFucNAc		2.07	

[a] PSs were either reduced with carbodiimide by the method of Taylor and Conrad (21) or were untreated (unreduced).

[b] HF indicates 49% aqueous HF at room temperature for 24 h alone; HF-HCl indicates the same procedure followed by 2 M HCl at 100°C for 3 h.

RESULTS

Isolation of IT-3 high-molecular-weight PS. An average of 75 mg of IT-3 PS was recovered after growth of the IT-3 strain of *P. aeruginosa* in 14 liters of the chemically defined medium of Terleckyj et al. (22). Immunodiffusion analysis of the isolated PS and the O side chain of IT-3 LPS demonstrated a line of identity when these two antigens were reacted with rabbit antiserum to whole organisms (Fig. 1). Molecular size analysis by high-performance liquid chromatography showed a single peak of material with an apparent molecular weight of 1.3×10^5, as determined from a standard curve plotting the $\log_{10}$ molecular weight of dextran polymers versus elution volume. The O polysaccharide side chain prepared from the LPS had an apparent molecular weight of 0.3×10^5 by this method.

Chemical characterization of IT-3 PS. Biochemical analysis of IT-3 PS for protein, nucleic acids, lipids, and 2-keto-3-deoxyoctulosonic acid showed only low-level contamination with protein and nucleic acid (0.3 and 0.5% of total weight, respectively) and no detectable lipid or 2-keto-3-deoxyoctulosonic acid. IT-3 PS did not gel the *Limulus* amoebocyte lysate at a concentration of 10,000 ng/ml, but IT-3 LPS caused gelation at a concentration of 10 ng/ml. Carbohydrate analysis by traditional means was not possible because of the reported occurrence of unique dideoxyhexosamine and diaminouronic acid constituents in IT-3 O side chains (5, 6). Analyses for the water content of material indicated a gain of approximately 20% in the weight of IT-3 PS freshly removed from a lyophilizer when compared with the weight of the same material after storage at −20°C for 1 week over silica gel.

Monosaccharide constituents of IT-3 PS and LPS. Knirel et al. (5, 6) have reported that the O polysaccharide side chain

TABLE 2. Antibody response of rabbits and mice to IT-3 PS and O side chains

Animals	Immunogen	Antibody level (μg/ml)[a]	
		Preimmune	Postimmune
Rabbits	PS	1.1 ± 0.4	23.2 ± 7.3
Mice (C3H/FeJ)	PS	0.9 ± 0.3	8.1 ± 6.2
	O side chains	0.9 ± 0.4	1.0 ± 0.3

[a] Values represent the geometric mean ± standard deviation of three rabbits or 10 mice.

isolated from the LPS of the Lanyi O:3(a),c serotype strain of *P. aeruginosa* is composed of a trisaccharide repeating unit consisting of 2,3-(1-acetyl-2-methyl-2-imidazolino-5,4)-2,3-dideoxy-D-mannuronic acid (DManImU), 2,3-diacetamido-2,3-dideoxy-L-guluronic acid [LGul(NAc)$_2$], and 2-acetamido-2,6-dideoxy-D-galactose (DFucNAc). This strain is reported to be serologically identical to the Fisher IT-3 strain. Thus, we expected these monosaccharides to be present in the IT-3 high-molecular-weight PS based on its serological identity with IT-3 O side chains. We found that both IT-3 PS and O side chains that were reduced with carbodiimide and then hydrolyzed with HF yielded a unique peak on gas-liquid chromatography with an R_{glu} of 1.03, and a second peak that co-chromatographed with authentic DFucNAc (R_{glu}, 2.07; Table 1). The former peak (R_{glu}, 1.03) was present at a level of only 1% in non-carbodiimide-reduced samples of IT-3 PS, suggesting that this peak was composed of either or both the DManImU and LGul(NAc)$_2$ components. The DFucNAc component was present in equal amounts in both reduced and nonreduced samples. The portion of reduced material that was then further hydrolyzed with 2 M HCl after HF hydrolysis showed a loss of the first peak (R_{glu}, 1.03) after the HCl hydrolysis (Table 1). This finding is consistent with the interpretation that the first peak was composed of either or both of the uronic acid moieties, since they are labile to 2 M HCl. Further support for the correctness of our gas-liquid chromatographic identification of these unique monosaccharides is provided by preliminary ^{13}C nuclear magnetic resonance analysis of the IT-3 PS and the O side chain. This analysis establishes a similarity between the nuclear magnetic resonance spectra of IT-3 PS and the O side chain and the nuclear magnetic resonance spectra reported by Knirel et al. for the Lanyi O:3(a),c O PS.

Animal toxicity. Mice (21 g) injected i.p. with 500 μg of IT-3 PS, and guinea pigs (350 g) injected i.p. with 2 mg of IT-3 PS, demonstrated normal weight gains and no evidence of systemic toxicity over a 2-week period. Three rabbits, each given 75 μg of IT-3 PS per kg, showed an aggregate temperature rise of 0.1°C over 3 h, indicating the lack of pyrogenicity of the IT-3 PS. Together, these studies indicate the safety of the IT-3 PS as assessed by standard toxicity and pyrogenicity assays.

Immunogenicity of IT-3 PS. Rabbits given a 2-week immunization course of intravenous injections of IT-3 PS had increased antibody levels to this antigen, as measured in the radioactive antigen binding assay (Table 2). Similarly, C3H/FeJ mice given three i.p. 50-μg doses of IT-3 PS at 5-day intervals had increased antibody levels 5 days after the final injection (Table 2). No increase in antibody level was seen in mice given three 50-μg doses of IT-3 LPS O side chains (Table 2).

Active protection of mice. Outbred CD-1 mice immunized with a single 50-μg dose of IT-3 PS had increased survival after i.p. challenge with live cells as compared with nonimmune mice ($P < 0.01$ by the Fisher exact test) (Table 3). Inbred C3H/FeJ mice immunized with three 50-μg doses of IT-3 PS were protected against subsequent live challenge at the site of a fresh burn injury with graded inocula of IT-3 bacteria (Table 3). This protection was manifested by a greater than 1,000-fold increase in the LD_{50} of the challenge strain in the immunized mice when compared with the unimmunized mice.

Cross-protection to IT-5 *P. aeruginosa*. Previously, we have used the cross-protective ability of high-molecular-weight PS antigen as a means of determining whether low-level contamination of PS antigens with LPS could account for the immunogenicity observed in PS preparations (11). Levels of LPS as low as 0.01% in PS can be immunogenic (14, 15). However, this immunogenicity results only in serotype-specific protection. Therefore, if high-molecular-weight PS can elicit heterologous serotype (heterotype) protection at doses comparable to those that give homologous type protection, then an immunologically active component in PS other than LPS must be responsible for heterotype protection. We found this to be true for the IT-3 PS, which was capable of protecting mice against challenge with IT-5 *P. aeruginosa* (Table 4). The dose of IT-3 PS needed to elicit protection against IT-5 PS was comparable to the dose needed for homologous protection (Tables 3 and 4). Low doses of IT-3 LPS induced homologous protection only (Table 4). Doses of LPS equivalent to those of PS were

TABLE 3. Protection of mice against *P. aeruginosa* IT-3 challenge after active immunization with IT-3 PS

Mouse strain	Status	Type of infection	Outcome		Significance
			No. of survivors/no. challenged	LD_{50}	
CD-1	Immune[a]	i.p.[b]	19/20		$P < 0.01$[c]
	Nonimmune		2/20		
C3H/FeJ	Immune[d]	Burn[e]		8.5×10^7	Non-overlapping
	Nonimmune			5.4×10^4	99% confidence intervals of LD_{50}[f]

[a] Received a single 50-μg i.p. injection of IT-3 PS 7 days before challenge.
[b] Received 4×10^7 CFU of IT-3 PS organisms.
[c] Calculated by the Fisher exact test.
[d] Received three 50-μg i.p. injections at 5-day intervals (controls received equivalent doses of bovine serum albumin on an identical schedule); challenge was 5 days after final immunization.
[e] Serial 10-fold dilutions of IT-3 organisms were injected subcutaneously at the site of a fresh, 10-s alcohol flame burn (2.5 by 2.5 cm); five mice were injected per dilution.
[f] LD_{50} and 99% confidence intervals were determined by the Spearman-Karber method (3).

TABLE 4. Protection of CD-1 mice against challenge with IT-3 and IT-5 *P. aeruginosa* (PA) after immunization with IT-3 PS and LPS

Immunogen	Dose[a] (μg)	% Survivors[b] after challenge with:	
		IT-3 PA	IT-5 PA
IT-3 LPS	0.01	80	0
	50	100	50
	100	100	80
IT-3 PS	50	90	80
	100	100	70
Saline		0	0

[a] A single injection was given i.p.
[b] $n = 10$; a survival rate of >60% represents significant protection ($P < 0.05$ by a Fisher exact test).

required to produce heterotype protection. These data strongly suggest that type-specific LPS is not a major immunogenic component in IT-3 preparations.

DISCUSSION

Our results indicate that an immunogenic, nontoxic, high-molecular-weight PS can be isolated from the IT-3 strain of *P. aeruginosa* in a manner analogous to that previously reported for IT-1 and IT-2 strains (12, 13, 15, 16). IT-3 PS has properties indicating that it is a high-molecular-weight form of the IT-3 LPS O side chain. The serological and chemical identities of the IT-3 PS and O side chains strongly support this conclusion. The different molecular weights of IT-3 PS and O side chains are likely responsible for their distinct immunological properties.

In contrast to IT-1 and IT-2 PSs, which contained arabinose and galactose in addition to monosaccharides associated with the LPS (15, 16), IT-3 PS contained no monosaccharides other than those also found in the IT-3 O side chain. As in the case of IT-1 and IT-2 PS, media components were excluded as contaminants of IT-1 PS because of our use of a chemically defined medium (22) that contained only low-molecular-weight components. Arabinose and galactose may represent components of a bacterial arabinogalactan that copurifies with the high-molecular-weight PS of IT-1 and IT-2 *P. aeruginosa*. Alternatively, the organism may synthesize the O side chain components on a molecule that also contains arabinose and galactose. The lack of these extra sugars in the IT-3 PS preparations indicates that this strain either does not make the arabinogalactan material or does not attach arabinose and galactose to the side chain. Despite the absence of these extra sugars, IT-3 PS is of sufficient molecular size to be immunogenic.

Our inability to completely characterize the monosaccharide constituents of IT-3 PS is due to the apparent occurrence of unique sugars that can only be identified by means not available in our laboratory. We have, however, inferred the presence of DManImU and LGul$(NAc)_2$ from our chemical results based on findings published from other laboratories on the O polysaccharide side chain structures of *P. aeruginosa* strains serologically identical to the Fisher IT-3 strain (5, 6). More detailed chemical and structural analyses of the IT-3 PS are in progress, and preliminary ^{13}C nuclear magnetic resonance data confirm the structural similarities of IT-3 PS and the IT-3 O side chains. The exact monosaccharide constituents still await characterization.

Isolation of the IT-3 PS extends the concept that *P. aerugionsa* strains produce a high-molecular-weight O side chain with vaccine potential. It is not presently known whether the PS material we are isolating is linked to native LPS and released in the course of our purification procedure, or whether IT-3 PS is produced as a separate molecule during actual bacterial growth. In either case, two striking features of *P. aeruginosa* high-molecular-weight PSs are their low toxicity and good immunogenicity in animals (11, 14, 15) and humans (12, 17). As demonstrated once again in this study, if conditions can be established for isolating nontoxic, immunogenic, high-molecular-weight PS from serotypes of clinically relevant strains of *P. aeruginosa*, a comprehensive multivalent vaccine should be feasible. The component PS antigens of such a vaccine are likely to be effective immunogens, based on the established role of serotype-specific immunity in the prevention of *P. aeruginosa* disease (18, 19).

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IN VITRO T CELL-MEDIATED KILLING OF *PSEUDOMONAS AERUGINOSA*

I. Evidence that a Lymphokine Mediates Killing[1]

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Previous studies have demonstrated *in vivo* that T cells can provide protective immunity, in the absence of antibody, against infection with the extracellular Gram-negative bacterium Immunotype 1 (IT-1) *Pseudomonas aeruginosa*. We established an *in vitro* system in which immune T cells, after re-exposure to bacterial antigens and to macrophages, secrete a product that kills the bacteria. Although macrophages are required for *in vitro* killing, they function neither as antigen-presenting nor as phagocytic cells in this system. T cells from animals immunized against a different *P. aeruginosa* immunotype will not kill IT-1 organisms; but the supernatants produced by IT-1 immune T cells after exposure to macrophages and IT-1 *P. aeruginosa* organisms are nonspecifically effective in killing unrelated bacteria. Because the supernatants from immune T cells lose their bactericidal properties upon minimal dilution, we conclude that if this mechanism is active *in vivo*, it must play a role in local immunity.

Protective immunity to extracellular bacteria has traditionally been attributed to the activity of antibody, complement, and phagocytic cells. Other than functioning as helper cells for antibody responses, T cells have been thought to be of little importance in such immunity. T cells do play a critical role in resistance to intracellular bacteria, and analysis of this resistance has provided new insights into the understanding of basic immune mechanisms (reviewed in Reference 1).

We have been studying protective immunity to the extracellular Gram-negative bacterium, *Pseudomonas aeruginosa* (*P. aeruginosa*). These bacteria contribute significantly to the morbidity and mortality of immunosuppressed patients, burn patients, and children with cystic fibrosis. Most studies of protective immunity against *P. aeruginosa* have focused on the role of antibody directed against the bacteria or their toxic products (2, 3). Few studies have examined the importance of T cells in protection against these bacteria.

We have identified an inbred mouse strain, BALB/c, that is incapable of generating an antibody response to low doses of a high m.w. polysaccharide (PS)[2] antigen isolated from broth cultures of *P. aeruginosa* (4). BALB/c mice immunized with low doses of PS could not resist challenge with the live organism any more effectively than unimmunized control mice. On the other hand, mice from another inbred strain, C3H/ANF, did produce antibody after low dose PS immunization, and when compared to unimmunized control mice, were protected against live bacterial challenge.

BALB/c mice that received the cytotoxic agent vinblastine sulfate at the time of low dose PS immunization failed to produce antibody, but they were protected against live bacterial challenge. The protective immunity observed in these mice was T cell-mediated (5).

To understand the mechanisms by which T cells participate in the destruction of extracellular bacteria, we established an *in vitro* system in which immune T cells kill *P. aeruginosa*. We now describe this system and define the mechanism by which T cells kill these extracellular bacteria.

MATERIALS AND METHODS

Bacteria. Fisher-Devlin immunotypes 1 and 4 (IT-1 and IT-4) of *P. aeruginosa* (originally provided by M. Fisher, Parke-Davis Co., Detroit, MI) were grown overnight in 50 ml trypticase-soy broth. Bacteria from this overnight growth were inoculated into 20 ml of fresh broth to obtain a relative optical density of 0.05 OD units (35 Spectrophotometer, Perkin-Elmer Corporation, Coleman Instruments Division, Oak Park, IL), and were allowed to grow to a density of 0.20 OD units. These bacteria were then harvested, washed once in 0.15 M NaCl, and resuspended to a concentration of 1 O.D. unit, which routinely contained 2 to 3 × 10^9 colony forming units of *P. aeruginosa*. The bacteria were then diluted in medium and added to microwells to assess *in vitro* killing. *Staphyloccocus aureus* (American Type Culture Collection [ATCC] 25923), *E. coli* (ATCC 25922), and a strain of *P. aeruginosa* highly resistant to gentamicin were obtained from stocks maintained in the Bacteriology Laboratory of The Jewish Hospital of St. Louis, St. Louis, MO.

Mice. BALB/c mice were obtained from the Animal Facility of The Jewish Hospital of St. Louis, and from Cumberland View Farms, Clinton, TN.

Cell separation. T cells were prepared according to Wysocki and Sato (6). Briefly, 3 × 10^7 spleen cells from BALB/c mice were placed for 70 min on petri dishes that had been precoated with 50 μl affinity-purified goat anti-mouse immunoglobulin (Ig) antiserum (Gateway Immunosera, St. Louis, MO). The nonadherent cells were collected and placed for 70 min on a second anti-mouse Ig antiserum coated petri dish. The nonadherent cells collected from this second cycle of adherence routinely contained fewer than 5% cells that reacted with

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[2] Abbreviations used in this paper: ATCC, American Type Culture Collection; IT-1, Immunotype 1; IT-4, Immunotype 4; PS, high m.w. polysaccharide isolated from *P. aeruginosa* broth cultures.

fluorescent goat anti-mouse Ig antiserum. The response of the nonadherent cells to the T cell mitogen concanavalin A (Con A; Pharmacia Fine Chemicals, Piscataway, NJ) was equivalent to the response of unseparated cells, whereas their response to the B cell mitogen lipopolysaccharide (LPS), obtained from *Salmonella enteriditis* (Difco, Detroit, MI) was routinely reduced by at least 75% when compared to the response of unseparated spleen cells. After the second cycle of adherence, nonadherent cells were incubated overnight at 37°C in 20 ml tissue culture flasks in a 5% CO_2 atmosphere, and the nonadherent cells from this overnight incubation were harvested and washed. Fewer than 1 of 300 of these cells were nonspecific esterase stain-positive.

Macrophages were obtained by lavaging the peritoneum of non-immune BALB/c mice with Hanks' balanced salt solution (HBSS). These resident cells were allowed to adhere overnight in microwells, and the nonadherent cells were then washed free the next day with warm medium. The remaining adherent cells were routinely 93 to 95% nonspecific esterase-positive.

T cells were depleted from immune spleen cell populations by incubation for 1 hr at 4°C in a monoclonal anti-Thy-1.2 antibody (diluted 1/250 in cytotoxicity medium) followed by a 45-min incubation at 37°C in low tox rabbit complement diluted 1/10. The reagents used in these T cell-depleting steps were obtained from Accurate Scientific and Chemical, Westbury, NY. The efficiency of the depletion is indicated in the legend to Table I.

Reagents. The bacterial PS used in these studies were prepared by described methods (5, 7). Vinblastine sulfate is a product of Eli Lilly Co., Indianapolis, IN.

Tissue culture medium. RPMI 1640 medium (GIBCO, Grand Island, NY) was supplemented with 2.0 mM L-glutamine, 25 mM HEPES buffer (GIBCO), 50 μg/ml gentamicin sulfate (Schering Corp., Kenilworth, NJ), and 10% heat-inactivated fetal calf serum (KC Biological, Inc., Lenexa, KS), which had been absorbed six times with 10^9 *P. aeruginosa*/ml to remove any antibody to these bacteria that might be present in the serum. Before absorption, no antibody could be detected in this serum by a radioimmunoassay that detects antibody to the PS antigens (8).

Assay of bacterial killing. T cell-enriched lymphocyte populations collected from overnight incubation of doubly "panned" spleen cells were washed and placed in antibiotic-free tissue culture medium at a concentration of 4×10^6/ml. Then 0.1 ml of these cells was placed in flat-bottomed microculture wells (Falcon 3072, Becton-Dickinson Labware, Oxnard, CA) that either did or did not contain adherent cells from the peritoneal lavages of non-immune mice. Live *P. aeruginosa* (1 to 2×10^2) in a volume of 0.1 ml of tissue culture medium were then added to the wells containing various combinations of cells and/or medium. Unless otherwise indicated, the bacteria used in all experiments were Fisher-Devlin IT-1. After a 4-hr incubation at 37°C in a moist 5% CO_2 atmosphere, the wells were pipetted vigorously and then a 50-μl sample was removed and plated on trypticase-soy agar. A second 50-μl sample was removed, diluted 1/10 in HBSS, and plated on agar. The next day, colonies on each plate were counted and the number of surviving bacteria in each well was calculated. For those studies in which culture supernatants were used to assay killing, either 10^3 heat-killed or 10^2 live bacteria were added to the wells in which the supernatants were prepared. After 4 hr, the supernatants were collected and passed through a 0.45-μm sterile filter (Gelman, Ann Arbor, MI) to remove bacteria and cells. Then 0.1 ml of each supernatant was added to 0.1 ml of the bacteria (10^3/ml in tissue culture medium) in flat-bottomed microculture wells and bacterial survival was assessed after 4 hr incubation at 37°C in a 5% CO_2 atmosphere.

In all experiments, the percent of bacteria killed was determined by subtracting the mean number of bacteria surviving in experimental wells from the mean number surviving in wells containing only medium, dividing this difference by the mean number of bacteria surviving in the media control wells, and multiplying by 100.

Statistics. A one-way analysis of variance using an F test (9) was employed to analyze the difference between experimental groups. P values < 0.05 were considered significant.

RESULTS

In vitro T cell-mediated killing of P. aeruginosa. We first investigated the cell combinations and conditions required for *in vitro* T cell-mediated killing of *P. aeruginosa* IT-1. BALB/c mice were injected with 125 μg vinblastine (i.v.) and 10 μg IT-1 PS (i.p.); 6 to 7 days later, their spleen cells were harvested, enriched for T cells, cultured overnight, and then added to microwell cultures the next day. Resident peritoneal macrophages were harvested from non-immune mice, allowed to adhere to microwells overnight, and washed free of nonadherent cells. To microwells containing various cell combinations we added 2×10^2 bacteria and compared their survival after 4 hr incubation to bacteria surviving in wells containing only tissue culture medium. The combinations were immune T cells alone, resident macrophages alone, or both resident macrophages and immune T cells. We also studied bacterial survival in the presence of resident macrophages, immune T cells, and 10^8 heat-killed bacteria. Both macrophages and immune T cells had to be present to effect a significant ($p < 0.05$) reduction in viable bacteria, compared to wells containing only medium (Fig. 1). The number of bacteria remaining in wells containing only immune T cells or only macrophages did not differ significantly from wells containing medium only ($p > 0.30$).

In similar experiments, survival of bacteria in wells containing only resident macrophages was quite variable, but was always significantly greater than in the presence of resident macrophages and immune T cells. Furthermore, the number of viable bacteria in wells containing only immune T cells was always significantly greater than in wells containing both macrophages and immune T cells.

The addition of 10^8 heat-killed bacteria to macrophages and immune T cells did not further decrease bacterial survival, suggesting that 2×10^2 live bacteria are sufficient to stimulate the immune response. On the other hand, the observation that 10^8 heat-killed bacteria did not significantly increase bacterial survival provides evidence that the reduction in viable bacteria is not mediated by antibody or any other antigen-specific product. Such a huge excess of dead bacteria would be expected to adsorb and deplete any antigen-specific product in the medium, unless that product were present in great excess.

In the experiment shown in Figure 1, 2×10^2 bacteria had been added to each well. After 4 hr incubation, the

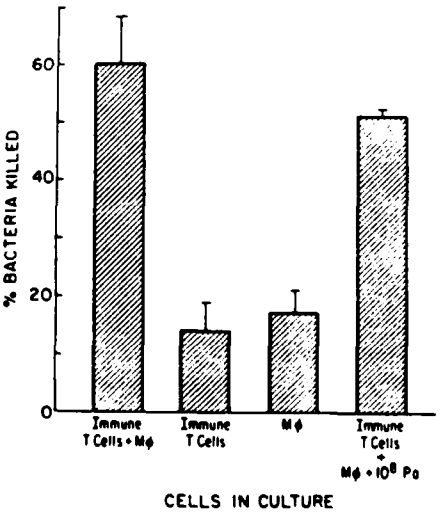

Figure 1. Killing of 2×10^2 *P. aeruginosa* (P.a.) in the presence of macrophages (Mϕ) plus immune T cells; immune T cells alone; macrophages alone; or macrophages, immune T cells, and 10^8 heat-killed *P. aeruginosa*. Control wells contained only tissue culture medium. Each point represents the mean ± SEM of four microwells.

wells with medium alone contained a mean of 532 bacteria, in contrast to a mean of 216 bacteria in wells with macrophages and immune T cells. Subsequent experiments indicated that bacterial killing, rather than growth inhibition, occurs in wells containing macrophages and immune T cells.

Comparison of killing by immune T cells and immune B cells. We next compared bacterial killing by T cell-depleted immune spleen cell populations with bacterial killing by B cell-depleted immune spleen cell populations. The data (Table I) indicate that, when compared with media controls, 60% of the bacteria in wells with macrophages and B cell-depleted populations were killed. Only 31% of the bacteria were killed by macrophages and T cell-depleted populations ($p = 0.01$). Thus, macrophages and T cell-enriched populations of immune spleen cells are more effective at killing bacteria than are macrophages and T cell-depleted populations.

Comparison of killing by immune and non-immune T cells. We next compared the ability of immune and non-immune T cells to promote bacterial killing (Table II). Wells containing non-immune T cells and peritoneal macrophages from non-immune mice contained 28% fewer viable bacteria than control wells. Wells with T cells from immunized mice and macrophages from non-immunized mice contained 63% fewer bacteria ($p = 0.02$, non-immune *vs* immune T cells). These observations are consistent with our previous results showing that non-immune BALB/c mice are highly resistant to *P. aeruginosa* infection and that this resistance can be enhanced by immunization with PS and vinblastine (4, 5).

Specificity of killing by T cells from mice immunized with PS and vinblastine. BALB/c mice were immunized with a PS isolated from Fisher-Devlin IT-4 *P. aeruginosa*, and 6 days later, their T cells were harvested and placed in tissue culture with macrophages from non-immune mice. We added 2×10^2 live IT-4 or IT-1 *P. aeruginosa* to these cultures and assayed bacterial survival 4 hr later. The results (Table III) show that macrophages and IT-4 immune T cells effectively kill IT-4 organisms (98% killed), although these macrophages and IT-4 immune T cells are ineffective in killing IT-1 organisms (21% killed). We conclude that activation of T lymphocytes for *in vitro* killing requires re-exposure to the homologous immunizing antigen.

Killing by supernatants from cultures of immune T cells and macrophages. To examine whether the observed killing resulted from the secretion by cells of a lethal product or from direct interaction of bacteria and cells, we assayed the ability of cell-free culture supernatants to kill *P. aeruginosa*. Macrophages and T cells were prepared as in previous experiments and five different groups of microwell cultures were set up (Fig. 2): 1) macrophages, immune T cells, and 10^3 heat-killed *P. aeruginosa*; 2) macrophages and immune T cells without bacteria; 3) immune T cells and 10^3 heat-killed *P. aeruginosa*; 4) macrophages and 10^3 heat-killed *P. aeruginosa*; and 5) 10^3 heat-killed *P. aeruginosa* in medium without cells.

TABLE I

Comparison of killing of P. aeruginosa by immune T cells vs immune B cells

Group	Cells in Final Culture	Bacteria Surviving in Well (% killing)	p Value[a]
1	None (media control)	189 ± 10^b (—)	<0.0001
2	Macrophages	173 ± 5 (8)	<0.0001
3	Anti-Thy-1.2-treated immune spleen cells + macrophages[c]	131 ± 14 (31)	0.01
4	Anti-Ig-treated immune spleen cells + macrophages[c]	76 ± 13 (6)	—

[a] This value expresses the significance of the difference between the number of bacteria surviving in wells containing anti-Ig-treated immune spleen cells and macrophages (group 4) and the number surviving in the given experimental group.

[b] Mean ± SEM of number of bacteria surviving in four microwells.

[c] Proliferative responses (± SEM) to T and B cell mitogens of the separated cells were: group 3: Con A 55,977 ± 5904, LPS 35,867 ± 1034; group 4: Con A 137,014 ± 9987, LPS 2,670 ± 372; unseparated spleen cells: Con A 218,508 ± 3503, LPS 60,470 ± 1509.

TABLE II

Killing of P. aeruginosa by T cells from immunized vs non-immunized mice

Cells in Culture	Number of Bacteria Surviving (% killing)	p Value[a]
None (media control)	836 ± 48^b (—)	<0.0001
Immune T cells and macrophages	308 ± 8 (63)	—
Non-immune T cells and macrophages	604 ± 44 (28)	0.0005

[a] This value expresses the significance of the difference between bacteria surviving in wells containing immune T cells and macrophages compared with the other experimental groups.

[b] Geometric mean ± SEM of bacteria surviving in four microwells.

TABLE III

Specificity of protection achieved by immunization with IT-4 PS and vinblastine

Cells in Culture	IT-1 Bacteria Surviving[a] (% killed)	IT-4 Bacteria Surviving[a] (% killed)
Mϕ	$3,400 \pm 138$ (15)[b]	$3,824 \pm 166$ (17)[c]
Mϕ + 4×10^5 T cells	$3,160 \pm 106$ (21)[b]	92 ± 14 (98)[c]
Mϕ + 2×10^5 T cells	$3,436 \pm 183$ (14)	$1,224 \pm 196$ (74)
Mϕ + 1×10^5 T cells	$3,954 \pm 190$ (1)	$3,464 \pm 114$ (25)
Media alone	$4,000 \pm 186$ (—)	$4,624 \pm 260$ (—)

[a] Mean ± SEM of bacteria surviving in four microwells.

[b] $p > 0.3$ for significance of difference in killing of IT-1 *P. aeruginosa* between wells containing macrophages (Mϕ) and wells containing Mϕ and 4×10^5 IT-4 immune *P. aeruginosa*.

[c] p value <0.0001 for significance of difference in killing of IT-4 *P. aeruginosa* between wells containing Mϕ and wells containing Mϕ and 4×10^5 IT-4 immune T cells.

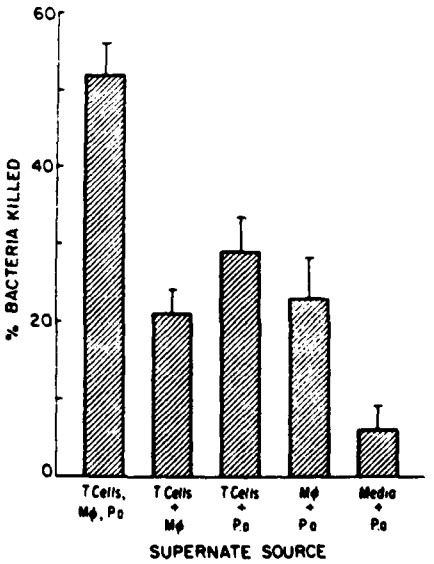

Figure 2. Killing of 1×10^2 *P. aeruginosa* (P.a.) by supernatants from microwells containing various combinations of immune T cells, macrophages (Mϕ), and 10^3 heat-killed bacteria. After 3 hr co-incubation of cells and bacteria, the supernatants were collected, transferred to microwells, inoculated with 1×10^2 live bacteria, and reincubated for 3 hr. Control wells contained fresh tissue culture medium. Each point represents the mean ± SEM of four microwells.

After 3 hr incubation, 0.1 ml of the supernatants from the five different cultures was placed in microculture with 10^2 live *P. aeruginosa* in 0.1 ml of medium. The control group contained fresh tissue culture medium inoculated with 10^2 live *P. aeruginosa*. After 3 hr incubation, the number of viable bacteria was determined for each supernatant group. When compared with the control, the supernatant from wells containing T cells, macrophages, and bacteria reduced the number of viable bacteria by 52%; this killing was significantly greater than that observed in any of the other groups ($p < 0.005$). Supernatants from wells containing T cells and macrophages killed 21% of *P. aeruginosa*, those from wells containing macrophages and bacteria killed 23%; those from wells containing T cells and heat-killed bacteria, 29%; and those from wells containing heat-killed bacteria but no cells, 6% of the *P. aeruginosa*.

There are two points of note in these results. First, bacterial killing in this *in vitro* system is mediated by a soluble product and does not require phagocytosis by macrophages. Second, maximal killing occurs with supernatants obtained from cultures containing T cells, macrophages, and bacteria, whereas supernatants from cultures containing T cells and macrophages but no bacteria produced significantly less killing ($p = 0.0003$).

Determination of which cell secretes the soluble bactericidal product. To determine whether the soluble bactericidal product was a lymphokine or a monokine, we conducted two separate experiments. To analyze which cell was responsible for the bactericidal effect observed in the presence of immune T cells, we assayed three groups of immune T cells (4×10^5/well) and two groups of macrophages.

The immune T cell groups were: 1) T cells that had been incubated previously for 3 hr with 4×10^3 macrophages and 10^3 heat-killed bacteria; 2) T cells that had not been incubated with either macrophages or bacteria; and 3) T cells that had been incubated for 3 hr with 4×10^3 macrophages without bacteria. The T cells were harvested from their respective cultures, washed, and placed in fresh culture medium with 10^2 live *P. aeruginosa*; bacterial survival was determined after 3 hr.

The two groups of macrophages were: 1) 4×10^3 macrophages that had been incubated with 4×10^5 immune T cells and 10^3 heat-killed bacteria, and 2) 4×10^3 macrophages that had not been exposed to T cells or bacteria. The macrophages were washed with warm medium to remove T cells and heat-killed bacteria, and then 10^2 live *P. aeruginosa* were added to the wells. Bacterial survival was assayed after 3 hr incubation at 37°C. A media control group was inoculated with 10^2 live bacteria, and bacterial survival was assayed at the end of 3 hr.

The results (Table IV) show that resident macrophages that had not been exposed to T cells and heat-killed bacteria (group 5) killed 46% of the bacteria when compared with control wells. Macrophages that had been exposed to immune T cells and heat-killed bacteria (group 4) killed 63% of the bacteria compared with the control, but this number did not differ significantly from the 46% killing seen in group 5 ($p = 0.07$). On the other hand, immune T cells that had not been exposed to macrophages or bacteria (group 2) killed 29% of the bacteria compared to the media controls, whereas T cells that had been exposed to macrophages and bacteria (group 1) killed 99% of the bacteria ($p < 0.0001$, group 1 *vs* group 2). These data indicate that the final bactericidal product resulting from the interaction of immune T cells and macrophages is a T cell lymphokine.

TABLE IV

Ability of T cells and macrophages to kill P. aeruginosa after previous incubation with and without heat-killed bacteria

Group	Cells in Final Culture	Bacteria Surviving[a]	p Value[b]
1	Immune T cells previously cultured with macrophages and heat-killed bacteria	8 ± 4 (99)	<0.0001 (2)
2	Immune T cells not previously cultured with macrophages or bacteria	456 ± 76 (29)	—
3	Immune T cells previously cultured only with macrophages	24 ± 8 (96)	<0.0001 (2)
4	Macrophages previously cultured with immune T cells and heat-killed bacteria	236 ± 32 (63)	0.07 (5)
5	Macrophages not previously cultured with T cells or bacteria	344 ± 50 (46)	—
6	Media alone (control)	640 ± 44 (—)	—

[a] Mean ± SEM of bacteria surviving in four microwells.
[b] This value expresses significance of difference between the particular experimental group and the group indicated within the parentheses.

A surprising finding was that immune T cells exposed to macrophages in the absence of bacteria could, upon subsequent exposure to live bacteria, reduce the number of viable organisms by 96% compared with media controls ($p < 0.0001$, group 3 *vs* group 6). This observation suggests that the critical interaction between macrophages and immune T cells that must occur to achieve optimal T cell bacterial killing does not involve presentation of antigen to T cells by macrophages. In fact, T cells do not have to be simultaneously exposed to macrophages and bacteria to be activated for bacterial killing, although exposure of T cells to macrophages is clearly required for the T cells to kill the bacteria to which they are subsequently exposed ($p < 0.0001$, group 1 *vs* group 2).

The reduction in viable bacteria in this experiment was so profound that it enables us to confirm that the reduction in bacterial survival observed in these *in vitro* experiments actually represents bacterial killing. We added 2×10^2 live bacteria to the cells in group 1 and only eight bacteria remained in these wells after 3 hr incubation.

Although this experiment suggests that the T cell secretes a bactericidal product, we sought to confirm this conclusion directly. We therefore again co-cultured resident peritoneal macrophages and immune T cells overnight, and the following day we separated the T cells from the adherent macrophages. After washing, each cell type was placed in separate microcultures with fresh media and 300 live *P. aeruginosa*. After 4 hr, these cultures were harvested and filtered to remove bacteria and cells. We then examined the ability of the different culture supernatants to kill *P. aeruginosa* (Table V). We also investigated whether the combination of T cell supernatant and macrophage supernatant produced synergistic killing.

Supernatants obtained from T cells that had been incubated overnight with macrophages, separated from those macrophages, and then exposed to live bacteria, killed 50% of the bacteria compared to a control well containing only tissue culture media. Macrophage supernatants, obtained from macrophages that had been exposed to T cells overnight and subsequently to live bac-

TABLE V

Ability of culture supernatants from isolated populations of macrophages or immune T cells to kill P. aeruginosa

Supernatant Source[a]	Bacteria Surviving (% killed)[b]	p Value[c]
T cells	513 ± 79 (50)	—
Macrophages	804 ± 71 (21)	0.02
T cells and macrophages[d]	653 ± 18 (36)	0.2
Media control	1017 ± 22 (—)	0.0008

[a] Supernatants were prepared by co-incubating macrophages and immune T cells overnight, separating and washing the two cell populations, exposing them to live bacteria for 4 hr, and then harvesting and filtering the supernatant. All cultures contained 200 μl of supernatant.

[b] Mean ± SEM of bacteria surviving in three microwells.

[c] This value compares the significance of differences between the bacterial survival in wells containing T cell supernatants and the bacterial survival in the other experimental groups.

[d] This group of wells contained 100 μl of macrophage supernatant and 100 μl of T cell supernatant.

TABLE VI

Ability of diluted supernatants from cultures of immune T cells, macrophages, and heat-killed bacteria to kill live P. aeruginosa

Supernatant Dilution	Bacteria Surviving (% killed)[a]	p Value vs Media Control	p Value vs ½ Dilution
Media	1,256 ± 146 (—)	—	0.0002
½	664 ± 12 (47)	0.0002	—
¼	824 ± 26 (34)	0.003	0.2
1/6	736 ± 42 (41)	0.0006	0.5
1/12	1,008 ± 128 (20)	0.06	0.01

[a] Mean ± SEM of bacteria surviving in four microwells.

teria, killed only 21% of the bacteria, significantly fewer bacteria than were killed by the T cell supernatants ($p = 0.02$). Adding macrophage supernatant to T cell supernatant did not significantly alter the killing by the T cell supernatant ($p = 0.2$, T cell supernatant *vs* combined T cell and macrophage supernatants), and if anything, served only to dilute and reduce the bactericidal effect of the T cell supernatant. This experiment therefore provides direct evidence that T cells secrete a bactericidal factor, the effect of which is not enhanced by macrophage supernatants.

Ability of different concentrations of supernatant to kill bacteria. To determine the potential physiologic role of the bactericidal product secreted by T cells, we asked whether this product is produced in concentrations that would be effective systemically *in vivo*. We compared killing at various dilutions of supernatant with the killing observed in media control wells and with the killing observed in wells with the highest supernatant concentration. Because live bacteria had to be added to the undiluted supernatants to evaluate killing, the most concentrated supernatant evaluated was 1/2. The results (Table VI) demonstrate that significant killing, in comparison to media controls, is achieved until the supernatant is diluted 1/12. At that point the killing is also significantly less than that achieved with the highest supernatant concentration (1/2 dilution). This observation suggests that the lethal component in the culture supernatants is not potent enough to be effective systemically, and if it does function *in vivo*, it functions, like other lymphokines, only over short distances.

Specificity of killing by culture supernatants. To investigate whether the bactericidal T cell product is specific in its activity, we collected supernatants from 3-hr cultures of immune T cells, macrophages, and heat-killed *P. aeruginosa*, and placed 100 μl in microwells containing approximately 10^2 colony-forming units of either *S. aureus*, *E. coli*, or the original strain of *P. aeruginosa* IT-1. In a separate experiment, we compared survival of our original strain of *P. aeruginosa* IT-1 with a strain of *P. aeruginosa* that is highly resistant to gentamicin. The latter experiment was performed to determine whether the killing we were observing was due to the overnight incubation of the T cells and macrophages in medium containing gentamicin. The results (Fig. 3) demonstrate that the killing is not specific. The supernatant was able to reduce the number of viable *S. aureus* and *E. coli*, compared with their respective media controls, even more effectively than it reduced the number of viable *P. aeruginosa*. The gentamicin-resistant strain was also killed by the supernatant, confirming that the killing was not related to the previous exposure of immune cells to that antibiotic.

DISCUSSION

In this report we described the *in vitro* conditions under which T cells can kill the extracellular bacterium *P. aeruginosa*. The killing is mediated by immune T cells, which must interact with macrophages *in vitro* before optimal bacterial killing can occur. Macrophages are not required, however, during the exposure of T cells to bacteria, indicating that in this system, macrophages do not function to present antigen to T cells. T cells from mice immunized with an unrelated *P. aeruginosa* PS are not stimulated to kill IT-1 *P. aeruginosa* *in vitro*. Our immunization protocol does not induce detectable antibody production *in vivo*, and *in vitro* B cells are significantly less effective than T cells in promoting bacterial killing. Soluble factors in supernatants from cultures of immune T cells that had been exposed *in vitro* to both macrophages and bacteria can kill the bacteria in a cell-free environment. These supernatants are nonspecific in their activity.

Our system represents a heretofore undescribed mechanism by which immune cells can kill bacteria. Described mechanisms of killing of extracellular bacteria include direct lysis by complement (10), phagocytosis by macro-

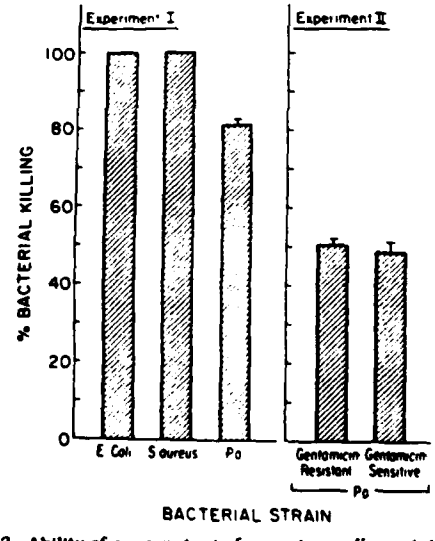

Figure 3. Ability of supernatants from microwells containing macrophages, immune T cells, and heat-killed *P. aeruginosa* (P.a.) IT-1 to kill other bacteria. In each experiment, the survival of bacteria in wells containing the culture supernatant and the given bacterial strain was compared to wells containing fresh tissue culture medium and that strain of bacteria. Each point represents the mean ± SEM of four microwells.

phages or granulocytes of opsonized bacteria (11), or destruction of antibody-coated bacteria by various types of killer cells (12). None of those mechanisms plays a role in the current system of killing.

Neither antibody nor complement is required to achieve killing in our assay. The BALB/c mice from which the immune T cells were derived do not produce detectable serum antibody in response to immunization (5), an observation that was reconfirmed in the present studies (data not shown). Fewer than 5% of the cells in the culture are B cells, and the addition of large numbers of heat-killed bacteria to the cultures, which would be expected to adsorb any antibody secreted by contaminating B cells, does not reduce the magnitude of the observed killing. Furthermore, the fetal calf serum used in the culture medium was heat-inactivated and adsorbed multiple times to ensure that no exogenous source of antibody or complement was present. The direct addition of B cells to this assay system yielded significantly less killing than was observed with B cell-depleted, T cell-enriched populations.

Two points about this killing mechanism are of particular interest. First, the final product that kills the bacteria is a lymphokine. Most studies of bacterial killing have focused on macrophage or granulocyte products, either lysosomal or extracellularly secreted (13, 14). Whereas T cell products such as interferon may be potent inhibitors of viral replication (15), the notion that T cells may secrete bactericidal products is a novel one.

Second, our observations define a new role for the macrophage in bacterial killing. In this system, some type of interaction between macrophages and T cells is required before T cells can respond to bacteria by secretion of a lethal product. In studies of bacterial killing, macrophages have traditionally functioned to present antigen to T or B cells and to phagocytize bacteria. Because in the current system the necessary interaction between T cells and macrophages can occur in the absence of antigen, macrophages are not functioning to present antigen to T cells. Furthermore, because bacterial killing occurs in the absence of macrophages, those cells do not have phagocytic function. Characterization of the essential role macrophages play in this killing mechanism requires further study.

How vinblastine functions in the elicitation of this immune response is, as yet, undefined. From preliminary evidence (unpublished observations), we suspect that by removal of a suppressor cell, this agent enhances proliferation of the clone of immune T cells that responds to the immunizing PS. This hypothesis is based on the observation that T cells from vinblastine-treated mice spontaneously proliferate *in vitro*, and that adoptive transfer of normal spleen cells to vinblastine-treated mice prevents the subsequent *in vitro* proliferation. It would appear that by expanding the clone of *P. aeruginosa*-immune T cells, vinblastine acts to magnify and make more observable the immune response that may already be occurring after immunization with the PS alone. This effect would be similar to that described for cyclophosphamide with sheep red blood cells used as an antigen (16).

In a previous study, we showed that T cells from mice given PS and vinblastine could, in the absence of specific antibody, adoptively transfer protective immunity to challenge with live bacteria (5). We can only speculate on whether the mechanism of *in vitro* T cell killing we describe here is the same as that involved in *in vivo* protection. It appears that if it functions *in vivo*, this mechanism of T cell killing must be active only in local immunity, at sites at which T cells, macrophages, and bacteria would congregate, and at which the lymphokine would be present in concentrations great enough to kill bacteria. The rapid loss of bactericidal activity with dilution of the lymphokine suggests that, like many other lymphokines, this bactericidal factor cannot function at long distances or within the bloodstream, where its activity might be diluted. T cell immunity to extracellular bacteria could then function in a complementary manner with antibody, which does promote destruction of bacteria that enter the circulation.

Studies in rats (17) and mice (18) have demonstrated that T cell immunity is critical in resistance to infection with another extracellular bacterial pathogen, *Bacteroides fragilis*. The pathologic features of infection with *B. fragilis*, characterized by the development of abscesses, are distinct from that of *P. aeruginosa*, and preliminary reports indicate that the T cell mechanism involved in killing of *B. fragilis* is complement-dependent and mediated by a non-secreted, antigen-specific T cell product (19). It appears that more than one T cell mechanism may be involved in resistance to infection with extracellular bacteria.

The importance of T cell immunity in resistance to infection remains to be determined. There are well-described clinical situations in which localized infection develops and progresses despite circulating antibody (20–22). Most relevant to *P. aeruginosa* are children with cystic fibrosis, who develop chronic, localized *P. aeruginosa* pulmonary infections despite their extraordinarily high levels of antibody to the infecting organisms. Although these high antibody levels prevent bacteremia, they do not prevent the recurrent exacerbation of lung disease associated with *P. aeruginosa* infection in cystic fibrosis patients. Whether a failure of local T cell immunity accounts for this persistent infection is of considerable interest and can now be readily evaluated by adaptation of this *in vitro* system to address the clinical problem.

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